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We identified molecules that are involved in the cross talk between the epithelial and mesenchymal components of the developing and involuting mammary gland. The EFGR protein was shown to mediate normal ductal morphogenesis by acting from the stromal cells on the development of the mammary epithelial ducts. Additional evidence was provided concerning the function of the Caspase 1 gene product during lobular-alveolar development and involution of the mammary gland after weaning. An interesting observation from this work was mammary epithelial cells that will succumb to apoptosis enter the cell cycle first. This observation implies that at least a subset of the redundant epithelial cells undergo activation before cell death. In preliminary work mammary glands from the MMP14 null mouse were shown to have less adipose tissue but ductal penetration of the fat pad and initial duct formation occurred by post-natal day 13. Further experimentation is required to determine if the reduction in adipose tissue is related to the wasting phenotype of these animals, or is induced by the MMP14 null epithelial cells. The practical outcome of these studies is a better understanding of mammary gland development before, during and after pregnancy, and during the process of involution.

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Introduction

Our research focus has been to better define the molecules that mediate epithelial-mesenchymal interactions during normal development and differentiation in the mammary gland, and to identify markers that define the transition from normal to abnormal growth and differentiation. This work has identified one mesenchymal (stromal) protein, the Epidermal Growth Factor Receptor (EGFR) as being critically important for the formation of ducts and branching morphogenesis by the mammary epithelial cells. Because deletion of this gene results in early post-natal lethality a tissue recombination model was utilized to follow mammary gland development in its absence. It was demonstrated that stroma lacking EGFR could not support mammary epithelial duct formation and branching.

In other work the role of an intracellular protease called Caspase 1 was examined and it was shown to influence mammary gland development during pregnancy and remodeling during involution. In animals that lacked Caspase 1 there was a delay in the development of the lobulo-alveolar glands during pregnancy and in the remodeling of the gland during involution. In other contexts, Caspase 1 is involved in processing the cytokine Interleukin-1 (IL-1). We have not observed a role for IL-1 in the mammary gland, but Caspase 1 may regulate the processing of other cytokines that have a role in mammary gland development during pregnancy and involution.

Lastly, in work that was initiated in January we tested the hypothesis that one extracellular matrix protease, MT1-MMP (MMP-14), functioned during mammary gland development. Using MMP-14 null animals created at the NIH, we had begun to examine early post-natal mammary development in the absence of MMP-14. Initial observations indicate that these animals had a stromal phenotype with less adipose tissue. Further experimentation is necessary to determine whether this phenotype is a direct result of the absence of MMP-14, or a result of the animal's failure to thrive. However, the preliminary work indicates that the precursor cells that form the ductal epithelium penetrate the primary mesenchyme to enter the fat pad in the absence of MMP-14, and there is rudimentary duct formation.

Body

Jane Wiesen was the Principle Investigator for this grant from its initiation in 1997 to December of 1999. I will summarize the data that she obtained during this period.

Organogenesis of the mammary gland depends on the interplay between the epithelial and mesenchymal compartments (Henninghausen and Robinson, 1998). Signal transduction between the mammary epithelial cells and the surrounding stroma and extracellular matrix direct the normal growth, differentiation, and morphogenesis of the mammary gland, as well as its involution after pregnancy (Talhouk et al, 1992; Sympson et al, 1994; Lund et al, 1996; Werb et al, 1996; Fata et al, 1999). Most breast tumors are epithelial in origin and their development depends on the alteration of the surrounding stroma (Barsky et al, 1982; Van der Hoof, 1988; Borsi et al, 1992; Wiedner et al, 1991; Wiedner et al, 1992; Ronnov-Jessen et al, 1995; Thomasset et al, 1998). There are many classes of molecules involved in this cross talk including growth and differentiation factors, proteases, extracellular matrix proteins, cytokine receptors, and adhesion molecules. Epithelial-mesenchymal interactions also have a crucial role in the development of breast tumors (Lochter & Bissell, 1995; Sympson et al, 1995; Masson et al, 1998; Thomasset et al, 1998; Sternlicht et al, 1999). Mammary cancers have strict environmental requirements and unless supplemented with growth factors and/or extracellular matrix they do not thrive outside of the mammary fat pad. Thus, the mammary epithelium and stroma have reciprocal interactions that direct their normal growth and differentiation, and contribute to neoplasia.

To investigate these interactions on a molecular level, mammary gland development in the Epidermal Growth Factor Receptor (EGFR) null mouse model was investigated (Miettinen et al, 1995; Sibilia & Wagner, 1995; Threadgill et al, 1995). One recent example of the role the EGFR has in epithelial-mesenchymal interactions is in the development of the palate. The EGFR is expressed by the epithelial cells in the palate and it upregulates ECM deposition and mesenchymal cell migration which leads to palate closure (Miettinen et al, 1999). In the absence of the EGFR palate closure does not occur and the pups have difficulty feeding. Because the

EGFR null animals die within a few days of birth, it is not possible to isolate mammary glands from pubescent or adult animals. Jane took advantage of a transplantation model where mammary glands are grown under the kidney capsule of immuno-compromised mice. Mammary glands from wild type mice recapitulate normal epithelial growth and ductal development while under the kidney capsule (Cunha et al, 1997). The results of this investigation indicated that expression of the EGFR was required in the stromal compartment to get normal ductal morphogenesis. However, EGFR expression was not essential for the lobulo-alveolar development that occurs during pregnancy, at least not in this experimental system. These results were published in 1999 in the journal Development. A copy of this article is found in Appendix II. Additional research examining aspects of EGFR signaling in epithelial-mesenchymal interactions in other systems is found in Appendix III, IV and V.

In other work Jane examined the role of the intracellular protease Caspase 1 in mammary gland development and involution. Caspase 1 is the founding member of a family of intracellular proteases that have essential roles in the activation or execution of apoptotic cell death (Earnshaw et al, 1999). Caspase 1 was originally identified based on its ability to cleave and activate IL-1 and is also called ICE (Interleukin-1 Converting Enzyme). Caspase 1 is upregulated during involution of the mammary gland (Boudreau et al, 1995; Lund et al, 1996). After weaning the excess mammary tissue undergoes apoptosis and is cleared (Lund et al, 1996). There is significant stromal alteration associated with the remodeling process including: upregulation of the extracellular matrix proteases, downregulation of their inhibitors, changes in the composition of the basement membrane, and a shift to adipose tissue deposition (Talhouk et al, 1992; Lund et al, 1996).

To investigate its role in mammary gland development, Jane used the Caspase 1 deficient mouse created at Merck Pharmaceuticals. These animals are viable, fertile and able to nurse their pups (Li et al, 1995; Kuida et al, 1995). However, a detailed examination of mammary gland development during pregnancy revealed subtle differences between Caspase 1 null and wild type animals. Lobular-alveolar development during pregnancy was delayed as a result of

alterations in the ratio of cell proliferation to cell death in the mammary glands of null animals. Additionally, the null animals exhibited delays in the natural involution and remodeling of the gland that takes place after weaning. In Caspase 1 null animals the redundant cells in the involuting glands exhibited delayed entry into the cell cycle. In a related study, contained in Appendix IX, Jane had determined that involuting mammary gland cells enter the cell cycle prior to apoptosis. Slower entry into the cell cycle in the Caspase 1 null cells delayed the apoptosis of redundant or obsolete cells during involution and increased the time required for remodeling the gland.

These results indicate a role for Caspase 1 in lobular-alveolar formation and the remodeling of the mammary gland during involution, but also raise several important questions. For example, what are the substrates of Caspase 1 during lobular-alveolar development and during involution? Does the retarded apoptosis observed in the Caspase 1 null animals result from its role as an Interleukin-1 Converting Enzyme? The role of IL-1 in the development of the mammary gland during pregnancy and involution should be investigated further. Additionally, other cytokine substrates for Caspase 1 exist and might be associated with mammary gland development during pregnancy and its involution after weaning (Gu et al, 1997; Ghayur et al, 1997). A draft manuscript of this study titled, "Mammary gland development and remodeling is altered in the Caspase-1 null mouse," is included in Appendix X of this report.

The cell cycle status of a cell determines whether it is in a resting or proliferative state. Activation-induced cell death occurs when signals received by a cell trigger it to go from a resting state, where it does not copy its DNA, to an activated state where it enters the DNA replication phase of the cell cycle and then dies. Activation-induced cell death is common in the immune system. This mechanism removes activated T cells, and may prevent autoimmunity by eliminating inappropriate responses to self-antigens (Shi et al, 1992; Green et al, 1992; Bissonnette et al, 1994). The myc gene is involved in the decision of activated lymphocytes, myeloid cells, and fibroblasts to proliferate or apoptose (Evan et al, 1992; Amati et al, 1993; Bissonnette et al, 1994; Hermeking and Eick, 1994; Hoffman and Liebermann, 1998). Recent

data indicates that in activated T cells, myc may induce expression of the Fas ligand, which triggers cell death (Brunner et al, 2000). Deregulation of the apoptosis-inducing function of myc can also contribute to lymphoma (Moroy et al, 1990; Gu et al, 1994). Cells that enter the replication phase of the cell cycle but then undergo apoptosis instead of proliferating are thought to die because they receive incomplete or conflicting signals (Hoffman and Liebermann, 1998). For example, the cell may be triggered to proliferate but may lack factors required to support its continued survival. Conversely, it may receive positive signals for proliferation while also receiving signals to stop proliferating and differentiate.

Apoptosis is a normal part of development and morphogenesis that shapes tissues and organs. Jane investigated whether the normal developmental apoptosis occurring during mammary gland involution required the cells to enter the cell cycle. Her preliminary results indicate that during involution the apoptotic cells enter the cell cycle prior to undergoing cell death. This data is contained in a draft manuscript in Appendix IX of this report titled, "Mammary epithelial cells enter the cell cycle prior to the onset of apoptosis during involution." The observation that these cells are undergoing activation before cell death raises many questions about what stimulates this process, and whether such stimuli would cause apoptosis of mammary epithelial cells at other stages of mammary gland development. If such stimuli were identified, it would be possible to test whether they could trigger apoptosis, or increase the apoptotic rate of rapidly proliferating tumor cells. Because the myc gene has been identified in many other systems as a regulator of activation-induced cell death, it is critical to determine if it is involved in mammary involution. Dr. Wiesen left to take a position with Ingenuity Systems before she could complete the research for the Caspase 1 manuscript and the manuscript on activation-induced apoptosis. This research will continue under the guidance of Dr. Zena Werb.

When I took over the responsibility for the grant, my interest was in studying the effect of extracellular matrix proteases found in the mesenchyme during mammary gland development. Preliminary data from other members of the Werb laboratory indicated that the matrix metalloproteinases (MMPs) and their inhibitors have important roles in epithelial-mesenchymal

interactions during development of the murine mammary gland. MMPs are a large family of secreted or membrane-bound enzymes that cleave extracellular matrix proteins and other substrates. Murine mammary gland development begins before birth when the gland invades the fat pad and forms a small bud of epithelial cells. This epithelial cell bud will grow into a tree-like structure after puberty, through the elongation and branching of the epithelial ducts (Henninghausen & Robinson, 1998). When drugs or synthetic compounds that inhibit MMP activity are given to young mice, mammary gland branching and elongation are inhibited (Lund et al, 1996; Werb et al, 1996; M. Sternlicht, manuscript in prepartation). Similarly, in transgenic animals that overexpress the human form of the naturally occurring MMP inhibitor, Tissue Inhibitor of Matrix Metalloproteinases (TIMP-1), there is decreased ductal branching and elongation (M. Sternlicht, manuscript in preparation). Animals with reduced expression of murine TIMP-1 exhibited decreases in the number and expansion of ducts (Fata et al, 1999). These studies with MMP inhibitors indicate that MMPs have critical roles in branching morphogenesis of the epithelial ducts to form the adult mammary gland.

The expression of two MMP genes is upregulated during branching morphogenesis. Both MMP-2 (Gelatinase A) and MMP-3 (Stromelysin-1) are upregulated during ductal development. Transgenic mice that over-express MMP-3 exhibit increased branching, whereas animals lacking MMP-3 expression show decreased branching (M. Sternlicht, manuscript in preparation). However, in neither case is the elongation of the ducts altered as compared to wild type animals. Conversely, mice lacking MMP-2 have shorter ducts compared with wild type animals, but branching does not seem to be affected (B. Wiseman, unpublished data). Neither gene product is essential for ductal development, although morphogenesis is altered in the absence of either gene product (M. Sternlicht, manuscript in preparation).

The MMP-14 (MT1-MMP) gene has been shown in other model systems to cleave the MMP-2 protein to its active form (Strongin et al, 1995; Cowell et al, 1998; Hiraoka et al, 1998). MMP-14 is expressed in a mouse mammary gland epithelial cell line (Tanaka et al, 1997). There have also been reports that MMP-14 is expressed in invasive breast carcinomas, and that this

expression may activate MMP-2 in the stroma (Pulyeva et al, 1997; Jones et al, 1999). We were interested in determining if animals lacking expression of MMP-14 had alterations in ductal morphogenesis similar to the MMP-2 null animals. The MMP-14 null animals have a variety of developmental problems caused in part by the reduction in collagen remodeling in the soft connective tissue ECM (Holmbeck et al, 1999). Some of these developmental defects include skeletal dysplasia, severe osteopenia and arthritis. As many as one third of null MMP-14 animals die before weaning and exhibit wasting. Those animals that survive past weaning do not mature sexually and most succumb to wasting between 50 and 90 days of age (Holmbeck et al, 1999).

To begin to analyze mammary gland development in these animals, we took mammary tissue from animals at 10 and 13 days of age. As agreed upon with our collaborators, we received the animals dead and on ice. Remarkably, the mammary gland tissue remains viable for at least 24 hours when stored on ice, and can be used for morphological and transplantation studies. However, it is not suitable for assays like in situ hybridization, because other body tissue has begun to breakdown. We prepared the mammary tissue for morphological analysis. When observed in whole mount preparations, both the null and wild type glands exhibited penetration of the fat pad by epithelial cells as well as rudimentary duct formation as expected at the ages examined (data not shown). Hematoxylin and eosin stained sections through the mammary glands obtained from these animals are shown in Figure 1. The mammary glands of the MMP14 null animals are significantly smaller than in their wild type littermates, but they seem to be proportional to the size of the animal. The most obvious difference between wild type and MMP-14 null glands is the amount of adipose tissue visible in sections (compare Figure 1C to 1D). The lack of adipose tissue may result from the general wasting of the null mice. To investigate this hypothesis we had planned to do some reciprocal transplantation of mammary epithelial cells obtained from either wild type or null animals into either wild type or null mammary stroma. These recombinations of mammary epithelial tissue and stromal tissue could then be transferred to the kidney capsule of an immunocompromised host animal and allowed to develop further (Wiesen et al, 1998). Our expectation was that if the phenotype of the null animals resulted from

malnutrition, there would be no difference in the development of glands made up of null epithelia or stroma combined with wild type stroma or epithelia, respectively. However, if MMP-14 activity was required for the development of adipose tissue, these recombinations might have less adipose tissue than a fully wild type mammary gland. Unfortunately, our collaborator was unable to provide sufficient animals for these experiments to be completed by the end of the grant period. However, this research project will be continued under the direction of Dr. Zena Werb.

Key Research Accomplishments

Jane Wiesen:

- Signaling through the Epidermal Growth Factor Receptor (EGFR) is required for mammary ductal growth and development in the mouse.
- Signaling through the EGFR is not required for murine mammary lobulo-alveolar development.
- During ductal morphogenesis the EGFR signal is required only in the mammary fat pad which is the stromal compartment.
- The stromal EGFR signals are essential for instructing the mammary epithelia during ductal growth and branching morphogenesis.
- During mammary gland involution the mammary epithelial cells enter the cell cycle prior to undergoing apoptosis.
- Caspase 1 is expressed in the mouse mammary gland beginning at day 9 of pregnancy and continuing post-partum during lactation and involution.
- Caspase 1 is not expressed in mammary glands during development or in virgin glands.
- During involution of Caspase 1 null mammary glands, the entry of the epithelial cells into the cell cycle, which proceeds apoptosis, is delayed.
- Involuting mammary glands of Caspase 1 null mice exhibit an increased rate of replacement of epithelial cells with adipocytes when compared to wild type mice.
- Lactation is normal in Caspase 1 null mice.
- Lobular-alveolar development is delayed in Caspase 1 null mice as a result of alterations in the rate of proliferation and apoptosis of progenitor cells in the mammary gland of pregnant animals.

Key Research Accomplishments

Julie Rinkenberger:

- MMP14 is not required for invasion of the primary mesenchyme or fat pad by precursor mammary epithelial cells.
- MMP14 null animals have less adipose tissue in day 10 and day 13 mammary glands than their wild type littermates.
- MMP14 null animals begin to form rudimentary ducts in the mammary gland by 13 days of age.

Reportable Outcomes

Manuscripts, abstracts and presentations

1) Wiesen, J. F., J. S. Mudgett & Z. Werb. Perturbation of mammary gland development in ICE knockout mice. Poster presented at the Programmed Cell Death meeting at Cold Spring Harbor Laboratories in Cold Spring Harbor, New York, September 17-21, 1997.

2) Wiesen, J. F., P. Young, Z. Werb & G. R. Cunha (1998) Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. Development **126**, 335-344.

3) Hom, Y. K., P. Young, J. F. Wiesen, P. J. Miettinen, R. Derynck, Z. Werb & G. R. Cunha (1998) Uterine and vaginal organ growth requires epidermal growth factor receptor signaling from stroma. Endocrinology **139**, 913-921.

4) Sebastian, J., R. G. Richards, M. P. Walker, J. F. Wiesen, Z. Werb, R. Derynck, Y. K. Hom, G. R. Cunha & R. P. DiAugustine (1998) Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. Cell Growth & Differentiation **9**, 777-785.

5) Cunha, G. R., J. F. Wiesen, Z. Werb, P. Young, Y. K. Hom, P. S. Cooke & D. B. Lubahn (2000) Paracrine mechanisms of mouse mammary ductal growth. Advances in Experimental Medicine and Biology **480**, 93-97.

6) Wiesen, J. F. & Z. Werb (2000) Proteinases, cell cycle regulation, and apoptosis during mammary gland involution (minireview). Molecular Reproduction and Development **56**, 534-540.

7) Rinkenberger, J. L., M. D. Sternlicht, B. S. Wiseman, M. Sciabica, K. Holmbeck, J. Wiesen, H. Birkedal-Hansen & Z. Werb. Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in the stroma regulate mammary ductal morphogenesis. Poster presented at the Era of Hope meeting in Atlanta, Georgia, June 8-11, 2000.

8) Rinkenberger, J. & Z. Werb (2000) The labyrinthine placenta. Nature Genetics **25**, 248-250.

9) Wiesen, J. F. & Z. Werb. Mammary epithelial cells enter the cell cycle prior to the onset of apoptosis during involution. Manuscript in preparation.

10) Wiesen, J. F. & Z. Werb. Mammary gland development and remodeling is altered in the caspase-1 null mouse. Manuscript in preparation.

Employment or research opportunities applied for and/or received on experiences/training supported by this award.

Jane Wiesen is a Scientist at Ingenuity Systems.

Julie Rinkenberger is a Senior Scientist in Functional Genomics and Biotechnology at Bayer Pharmaceuticals, Berkeley, California.

Conclusions

This research has identified molecules that are involved in the cross talk between the epithelial and mesenchymal components of the developing and involuting mammary gland. The EFGR protein was shown to mediate normal ductal morphogenesis by acting from the stromal cells on the development of the mammary epithelial ducts. Although incomplete, additional evidence was provided concerning the function of the Caspase 1 gene product during lobular-alveolar development and involution of the mammary gland after weaning. An interesting observation from this and related work indicated that mammary epithelial cells that will succumb to apoptosis enter the cell cycle first. This observation implies that at least a subset of the redundant epithelial cells undergo activation before cell death.

Finally, in unrelated work, mammary glands from the MMP14 null mouse were shown to have less adipose tissue in the mammary gland. However, ductal penetration of the fat pad and initial duct formation occurred by post-natal day 13. Further experimentation is required to determine if the reduction in adipose tissue is related to the wasting phenotype of these animals, or is induced by the MMP14 null epithelial cells.

The practical outcome of these studies is that we have a better understanding of mammary gland development before, during and after pregnancy, and during the process of involution. We have identified a growth factor receptor (EGFR), an intracellular protease (Caspase 1), and an extracellular protease (MMP14) that have roles in the mammary gland during normal development. It is key to understand the normal development and involution of the mammary gland so that we can understand how these processes are perturbed in mammary cancer.

Identifying molecules that are involved in the apoptosis of mammary epithelial cells is the first step toward developing new drugs that exploit this mechanism against tumor cells. It is the shift in the ratio of tumor cells dying by apoptosis to those proliferating that results in their accumulation at a primary or metastatic site. Therefore, potential therapeutics that can shift the

balance toward apoptosis may be potent antagonists of primary and secondary tumors. Many commonly used chemotherapeutics are known to function in this manner (Lowe et al, 1993; Debatin, 2000). While this work does not directly address drug development based on this hypothesis, it has provided additional information about the mechanism of apoptosis during involution. As this work continues, it is hoped that new methods to exploit the apoptotic program in the mammary gland will be discovered.

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PERTURBATION OF MAMMARY GLAND DEVELOPMENT IN ICE KNOCKOUT MICE. Jane F. Wiesen[1], John S. Mudgett[2] and Zena Werb[1]. [1]Department of Anatomy, University of California, San Francisco CA 94143 and [2]Merck Research Laboratories, Rahway, NJ 07065

Interleukin-1β converting enzyme (ICE), which is involved in the processing and secretion of interleukin-1β, is a mammalian homologue of the *C. elegans* "cell death" gene *ced-3* that is required for apoptosis during development. ICE and its related caspase family members (mNedd-2, Mch2, CPP32, ICE rel II, ICE rel III, FLICE, ICE-LAP3, ICE-LAP6) are the "death effectors" in mammalian cells. In the mammary gland, ICE is expressed during involution following weaning, and in a mammary epithelial cell line undergoing apoptosis, while specific inhibitors of ICE can block induction of apoptosis *in vitro*. Using an ICE knockout mouse model we investigated the role of ICE in mammary gland development and function during pregnancy, lactation, and involution. During early pregnancy (6-12 d), the ICE knockout mammary glands had thinner ducts with fewer branches, and less alveolar budding. While mammary gland structure and function were comparable in the ICE knockout and wild type glands during late pregnancy and lactation, the knockout glands had altered remodeling during involution. During the early phase of involution (2-4 d), apoptosis occurred in the luminal cells and an accelerated infiltration of adipocytes was seen in the knockout glands compared to wild type glands. The patterns of cell proliferation, as measured by BrdU incorporation, and apoptosis were altered in the ICE knockout mouse as compared to the patterns seen in the wild type mice throughout pregnancy and involution following weaning. We conclude that the expression of the cysteine protease ICE, either via its interleukin-1β function or through its regulation of apoptosis, plays a role in the development of the mammary gland during pregnancy, and in the remodeling of the mammary gland during involution. We are using *in vitro* and transgenic approaches to examine the roles of ICE and IL-1β in regulating cell proliferation and apoptosis in the mammary gland to further address these isssues.

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Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development

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SUMMARY

Stromal-epithelial interactions are critical in determining patterns of growth, development and ductal morphogenesis in the mammary gland, and their perturbations are significant components of tumorigenesis. Growth factors such as epidermal growth factor (EGF) contribute to these reciprocal stromal-epithelial interactions. To determine the role of signaling through the EGF receptor (EGFR) in mammary ductal growth and branching, we used mice with a targeted null mutation in the *Egfr*. Because *Egfr*$^{-/-}$ mice die perinatally, transplantation methods were used to study these processes. When we transplanted neonatal mammary glands under the renal capsule of immuno-compromised female mice, we found that EGFR is essential for mammary ductal growth and branching morphogenesis, but not for mammary lobulo-alveolar development. Ductal growth and development was normal in transplants of mammary epithelium from *Egfr*$^{-/-}$ mice into wild-type (WT) gland-free fat pads and in tissue recombinants prepared with WT stroma, irrespective of the source of epithelium (Stroma^{WT}/Epi$^{-/-}$, Stroma^{WT}/Epi^{WT}). However, ductal growth and branching was impaired in tissue recombinants prepared with *Egfr*$^{-/-}$ stroma (Stroma$^{-/-}$/Epi^{WT}, Stroma$^{-/-}$/Epi$^{-/-}$). Thus, for ductal morphogenesis, signaling through the EGFR is required only in the stromal component, the mammary fat pad. These data indicate that the EGFR pathway plays a key role in the stromal-epithelial interactions required for mammary ductal growth and branching morphogenesis. In contrast, signaling through the EGFR is not essential for lobulo-alveolar development. Stimulation of lobulo-alveolar development in the mammary gland grafts by inclusion of a pituitary isograft under the renal capsule as a source of prolactin resulted in normal alveolar development in both *Egfr*$^{-/-}$ and wild-type transplants. Through the use of tissue recombinants and transplantation, we have gained new insights into the nature of stromal-epithelial interactions in the mammary gland, and how they regulate ductal growth and branching morphogenesis.

Key words: EGFR, Mammary gland, Stromal-epithelial interaction, Ductal morphogenesis, Mouse, Growth factor

INTRODUCTION

Mammary gland development consists of distinct stages, which include initial hormone-independent morphogenesis of the mammary buds and ducts during embryonic and perinatal life, hormonally regulated ductal development during puberty and lobulo-alveolar development during pregnancy. Each stage has distinct patterns of gene expression and specific hormonal requirements that influence the cross-talk between epithelium and mesenchyme to regulate development (Sakakura, 1987; Daniel and Silberstein, 1987). Mesenchymal-epithelial interactions are crucial during development of the embryonic mammary bud, when mammary mesenchyme induces the overlying ectoderm to form epithelial buds at day 12 of gestation (see Cunha and Hom, 1996 for review). Following formation of the mammary epithelial bud, reciprocal mesenchymal-epithelial interactions are critical for ductal growth and morphogenesis (Sakakura, 1987; Kratochwil, 1987). A number of growth factors are implicated as autocrine and paracrine mediators of mesenchymal-epithelial interactions in the mammary gland (Wysolmerski et al., 1995; Yang et al., 1995; Niranjan et al., 1995; Soriano et al., 1995; Imagawa et al., 1994; Vonderhaar, 1987; Coleman et al., 1988). Ligands of the epidermal growth factor receptor (EGFR) are believed to be particularly important downstream mediators of steroid hormone action in the mammary gland, acting locally to regulate mammary gland growth and development via stromal-epithelial interactions.

EGFR, a member of the ErbB/type 1 family of receptor tyrosine kinases, can form homodimers or heterodimers with the other family members: ErbB2, ErbB3 and ErbB4 (Earp et al., 1995). Multiple ligands bind to the EGFR including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF (HB-EGF) (Pinkas-Kramarski et al., 1997), and epiregulin (Komurasaki et al., 1997). These growth factors are synthesized as membrane-bound precursors that are cleaved by

proteases to release the active ligands. In the mouse mammary gland, the EGFR is expressed in the stromal cells surrounding the terminal end buds, in cap cells of end buds, and in adipocytes, myoepithelial cells and luminal epithelial cells (Coleman et al., 1988; Coleman and Daniel, 1990; DiAugustine et al., 1997). EGF stimulates growth of primary mammary epithelial cells from virgin or pregnant mice in vitro (Richards et al., 1982; Imagawa et al., 1985; Taketani and Oka, 1983). When slow-release implants containing EGF are introduced into growth-arrested mammary glands of ovariectomized mice, terminal end buds reappear in the zone around the implant (Coleman et al., 1988). To evaluate the role of signaling through the EGF receptor during ductal growth and branching morphogenesis in vivo, we used mice in which *Egfr* was inactivated by targeting exon 2 by homologous recombination (Miettinen et al., 1995). In this report, we have elucidated how signaling through the EGFR influences mammary ductal morphogenesis and lobulo-alveolar development. Moreover, we have determined that the stromal EGFR is a necessary component of the stromal-epithelial signaling interactions required for ductal growth and branching morphogenesis.

MATERIALS AND METHODS

Animals and determination of genotypes

$Egfr^{-/-}$ mice on a 129SV/Jx Swiss Black background (Miettinen et al., 1995) were produced by breeding pairs heterozygous for the targeted *Egfr* allele. Heterozygotes were genotyped by PCR (5′ primer: AGTAACAGGCTCACCCAACTGG, 3′ primer: CTACCCGCTTCCATTGCTCAGC). Amplification conditions were: denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes for 35 cycles. $Egfr^{-/-}$ mice were identified at birth by their open eyelids and short curly whiskers. There were no discernible differences between the heterozygotes and the wild-type homozygotes. Therefore, these animals are referred to as wild types throughout the grafting studies described. Intact female athymic nude mice (Balb/C) were purchased from Harlan (Indianapolis, IN).

Mammary gland dissection and transplantation

The #4 (inguinal) mammary glands (main duct, ductal branches and the entire fat pad) were removed from wild-type or $Egfr^{-/-}$ littermates on postnatal days 1-3. The glands were transplanted under the renal capsules of virgin female athymic mice (wild-type and $Egfr^{-/-}$ under contralateral renal capsules in the same animal) and grown for one month (n=14). To induce lobulo-alveolar development, some of the nude mouse hosts received a transplant of an adult pituitary gland under the renal capsule (Adler, 1986; Cunha et al., 1992). Animals were injected with bromodeoxyuridine (BrdU, 300 μg/mouse) 2 hours before grafts were harvested.

Cleared fat pads were prepared in 3-week-old female nude mice by removing the epithelial ducts as previously described (De Ome et al., 1959; Medina, 1996). Either wild-type or $Egfr^{-/-}$ epithelium was isolated from neonatal female mice by microdissection, transplanted into cleared glands and allowed to grow for 2 months (n=4). Animals were injected with BrdU (300 μg/mouse) 2 hours before harvest.

Tissue recombinants were prepared by surgically separating the neonatal fat pad from the epithelial rudiment. The primary main duct of the mammary rudiment was incubated with collagenase to remove adherent fibroblasts and placed onto a cleared fat pad in culture overnight to allow firm attachment of the epithelium and stroma as previously described (Cunha et al., 1997). The following tissue recombinants were produced: wild-type (WT) stroma + WT epithelium (epi) (n=26), WT stroma + $Egfr^{-/-}$ epi (n=36), $Egfr^{-/-}$ stroma + WT epi (n=31), and $Egfr^{-/-}$ stroma + $Egfr^{-/-}$ epi (n=22). Tissue recombinants were placed under the renal capsules of nude mice and permitted to grow for 1 month. Animals were injected with BrdU 2 hours before the grafts were harvested.

Morphologic analysis

For histology, the harvested grafts were placed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

For preparing whole mounts, grafts were fixed in acetic acid/methanol, the fat was cleared with xylene and the ductal trees were visualized by staining with either carmine or hematoxylin (Sympson et al., 1994).

Cell proliferation was determined by BrdU incorporated into the graft in vivo. BrdU (300 μg/mouse) was injected into the host animals 2 hours before harvesting of the grafts. Tissues and sections were protected from light exposure. BrdU incorporation was determined on paraffin sections by an anti-BrdU antibody (Zymed) and visualized

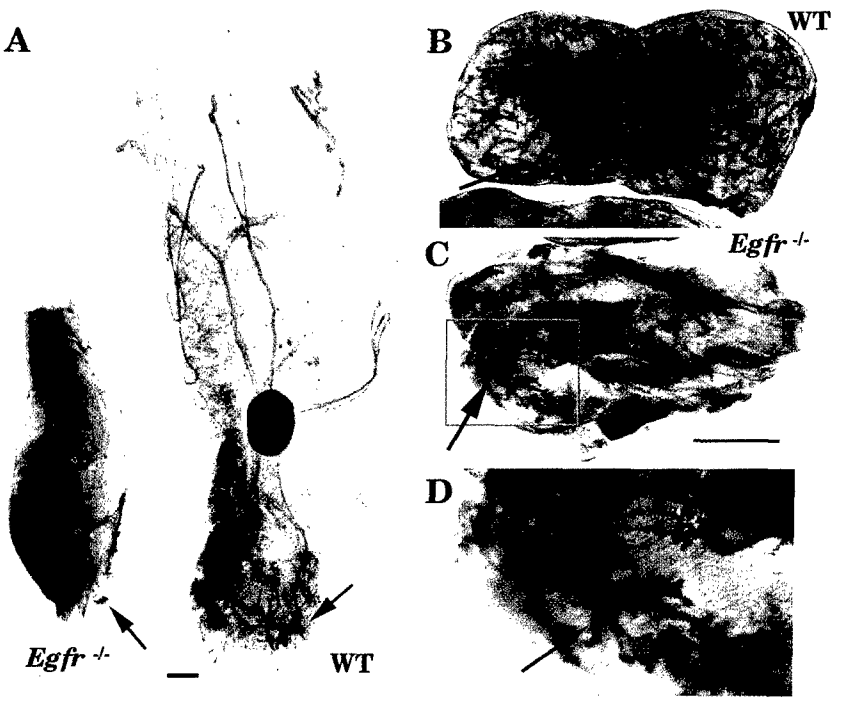

Fig. 1. (A) Whole-mount preparations of mammary glands from 11-day-old female mice. The epithelial ducts (arrows) infiltrate the $Egfr^{-/-}$ fat pad much less than in the wild-type gland. (B-D) Whole-mount preparations of mammary glands grown for one month under the renal capsule of a virgin athymic female mouse. The epithelial ducts barely infiltrate the fat pad in the $Egfr^{-/-}$ gland (C,D) compared to the wild type, which has undergone extensive ductal growth and branching to completely fill the fat pad (B). D is a higher magnification of the $Egfr^{-/-}$ ducts seen in the outlined green box in C. Bar, (A) 1 mm, (B,C) 2 mm, (D) 0.7 mm.

by diaminobenzidine. The slides were counterstained with hematoxylin.

To analyze apoptosis, paraffin sections of the grafts were stained using the Apoptag Fluorescein kit (Oncor) and cells counterstained with propidium iodide (Oncor). Cells undergoing apoptosis were visualized by fluorescence microscopy.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

β-casein gene expression was detected by isolating RNA from 6 paraffin sections of the mammary graft tissue according to the procedure of Weizsäcker et al. (1991). The reverse transcription reaction proceeded for 45 minutes at 48°C and PCR was carried out in the same tube using the Access RT-PCR system (Promega). PCR was performed with primers specific for the mouse β-casein gene (primer #1: AAGACCTTCTGCAGTACCTAGA; primer #2: CCTGTAATATAACTGAGAAGCA) using the following conditions: denaturation at 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 1 minute and 68°C for 2 minutes for amplification, and final extension at 68°C for 7 minutes. PCR products were run through a 1% agarose gel and visualized with ethidium bromide staining.

Morphometric analysis

Determination of total mammary gland and epithelial area was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Whole-mount preparations were imaged using a Leaf Lumina camera scanner (Leaf Systems, Scitex America Corp., Bedford, MA). The image files were opened in NIH Image. The total area of the gland defined by the perimeter of the fat pad was chosen by the 'Threshold' or 'Density Slice' command and a binary image was made. The area of this binary image was measured in NIH Image. The same commands were used to select epithelial area, but some of the selection had to be done manually due to contrast irregularities within the specimens. For each tissue recombinant type, at least 3-5 specimens were measured. Statistical differences in the values for each tissue recombinant type were assessed by an unpaired T-test done in the program Statview.

RESULTS

Initial development of the mammary anlage occurs in the *Egfr*$^{-/-}$ mouse

Although some *Egfr*$^{-/-}$ pups are born at a size similar to that of their wild-type littermates, the smallest mice (about 50% of the *Egfr*$^{-/-}$ mice) die within the first 24 hours. As a result of severe developmental defects in pulmonary and gastrointestinal epithelia (Miettinen et al., 1995), the surviving mice are growth retarded and, within a few days, typically have a body weight about one-third that of their wild-type littermates. The majority of *Egfr*$^{-/-}$ mice die within 3 days of birth. Based upon analysis of whole-mount preparations of #4 (inguinal) glands (data not shown), mammary gland development was equivalent at birth in both *Egfr*$^{-/-}$ mice and wild-type littermates suggesting that prenatal mammary development is normal in *Egfr*$^{-/-}$ mice. A few exceptional *Egfr*$^{-/-}$ mice survived until the age of puberty, when mammary ductal growth begins. By postnatal day 11 (Fig. 1A), the epithelial ducts have begun to migrate into the mammary fat pad in the wild-type gland, while very little ductal development has occurred in the *Egfr*$^{-/-}$ gland. These data indicate that, while mammary gland development is normal in *Egfr*$^{-/-}$ mice during the prenatal period, mammary ductal development is

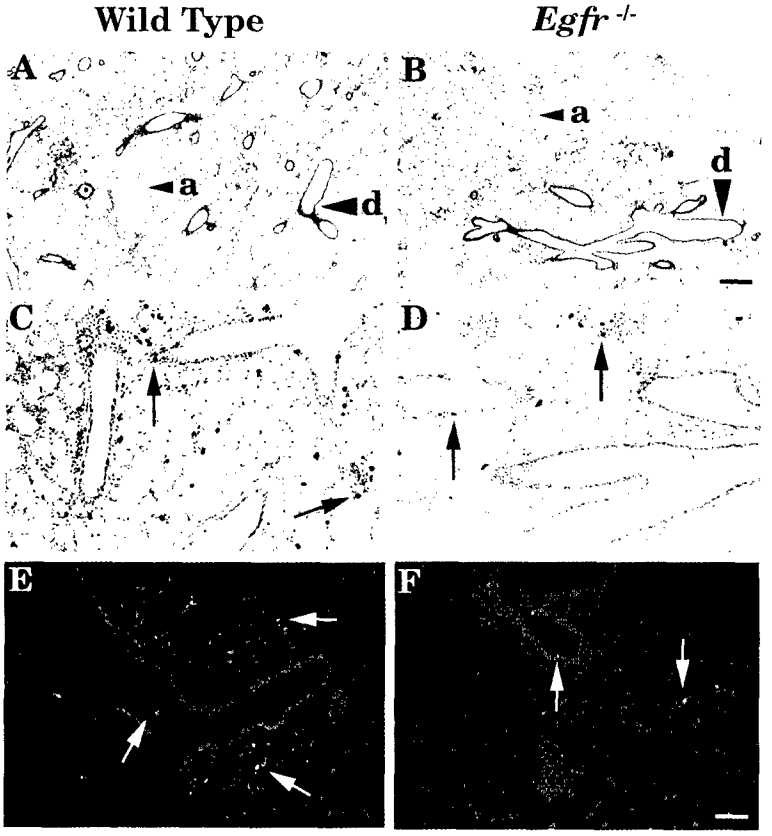

Fig. 2. Cell morphology, proliferation and apoptosis in wild-type and *Egfr*$^{-/-}$ mammary gland grafts. (A,B) The epithelial ducts (d) infiltrate through a mammary fat pad composed of adipose tissue (a). Very little ductal development is seen in the *Egfr*$^{-/-}$ grafts (B) compared to the wild-type grafts (A), and the few ducts present in the *Egfr*$^{-/-}$ gland have abnormally large, distended lumens. (C,D) Cell proliferation in mammary gland grafts as determined by BrdU incorporation. The proliferating cells are stained brown (arrows). There are fewer proliferating cells in both the stroma and the ductal epithelial cells in the *Egfr*$^{-/-}$ gland (D), compared to the wild-type gland (C). (E,F) Apoptosis in mammary gland grafts. Cells undergoing apoptosis are green (arrows). Since there are few ducts that have developed in the *Egfr*$^{-/-}$ gland (F), very little apoptosis is seen compared to the wild-type gland (E). The periductal fibroblasts are reduced in number around the few ducts present in the *Egfr*$^{-/-}$ gland (D,F). Bar, (A,B) 400 μm, (C-F) 100 μm.

impaired in surviving $Egfr^{-/-}$ pups during the prepubertal period.

Signaling through the EGFR is required for mammary ductal morphogenesis

To assess the full potential for mammary ductal morphogenesis that normally occurs during puberty, inguinal glands from $Egfr^{-/-}$ mice at postnatal days 1-3 (n=14) and glands from their wild-type littermates (n=14), were transplanted under contralateral renal capsules of athymic virgin mice. After 1 month, the epithelial ducts grew and filled the mammary fat pad in grafts of wild-type glands, while very little ductal development occurred in grafts of $Egfr^{-/-}$ glands (Fig. 1B-D). The few ducts in the $Egfr^{-/-}$ glands had abnormally large, distended lumens (Fig. 1D). Microscopically, the lack of ductal development in grafts of $Egfr^{-/-}$ mammary glands and the abnormally large lumens in the few existing ducts were clearly seen in H&E-stained sections (Fig. 2A,B). The $Egfr^{-/-}$ glands had very few proliferating cells (37% that of the wild-type glands) in either the stroma or the epithelial ducts, as determined by the labeling index with BrdU, after 1 month of growth in the nude mouse hosts (Fig. 2C,D). Wild-type glands had a few apoptotic ductal epithelial cells that were shed into the lumen, and both wild-type and $Egfr^{-/-}$ glands contained a small number of apoptotic cells in the stroma (1-2%) (Fig. 2E,F), when assessed after 1 month of growth in the nude mouse hosts. However, periductal fibroblasts were reduced in number around the few abnormal ducts that were present in the $Egfr^{-/-}$ glands compared to the wild-type glands (Fig. 2C-F). From these grafting experiments, we conclude that the $Egfr^{-/-}$ gland has a profound defect in ductal growth and morphogenesis, as indicated by the reduced number of ducts, the abnormally wide ducts, the decrease in epithelial and stromal proliferation, and the reduction in periductal fibroblasts.

The $Egfr^{-/-}$ epithelium is competent to undergo ductal development

Normal mammary epithelium contains stem cells that are competent to form ducts and secretory alveoli when transplanted into a cleared fat pad (DeOme et al., 1959). To determine whether the defect in ductal development in the $Egfr^{-/-}$ gland is due to the lack of EGFR signaling in the stroma, the epithelium, or both, we transplanted wild-type or $Egfr^{-/-}$ epithelium (n=4) into cleared fat pads of virgin female athymic mice and examined the ductal tree after 2 months. As seen in whole-mount preparations, the $Egfr^{-/-}$ epithelium grew and filled the fat pad to the same extent as the wild-type epithelium (Fig. 3). These experiments demonstrate that the $Egfr^{-/-}$ epithelium is competent to grow and undergo ductal morphogenesis, if given the correct developmental cues from the stroma, and suggest that the defect in ductal development seen in grafts of whole $Egfr^{-/-}$ glands may reside in the mammary fat pad.

Signaling through the stromal EGFR is necessary for ductal morphogenesis

To determine the contribution of the stroma in regulating epithelial proliferation and development in the $Egfr^{-/-}$ mice, neonatal mammary glands were surgically separated into fat pad and main duct and then recombined as described in Materials and Methods (Fig. 4). When the tissue recombinant contained wild-type stroma (Fig. 4A,D), ductal development proceeded regardless of the source of the epithelium (WT = 33% of the fat pad filled) versus $Egfr^{-/-}$ (23% filling) (group A, Fig. 5). This outcome is consistent with the results of transplantation of epithelium into cleared fat pads. However, when the tissue recombinants contained $Egfr^{-/-}$ stroma (Fig. 4B,C,E), there was very little ductal development regardless of the source of the epithelium (WT = 7% versus $Egfr^{-/-}$ = 5%) (group B, Fig. 5). The tissue recombinants that contained wild-type stroma (A) filled the pad pad to a greater extent ($P \leq 0.02$, n=3-5) than those prepared with $Egfr^{-/-}$ stroma (B) (Fig. 5). However, there was not a significant difference between the tissue recombinants within group A ($Stroma^{WT}/Epi^{WT}$ and $Stroma^{WT}/Epi^{-/-}$), or within group B ($Stroma^{-/-}/Epi^{WT}$ and $Stroma^{-/-}/Epi^{-/-}$) (Fig. 5). These data indicate that signaling through the stromal EGFR is essential for normal ductal growth and branching.

The number of proliferating cells was minimal in the tissue recombinants containing $Egfr^{-/-}$ stroma (0.5%), compared to the tissue recombinants prepared with wild-type stroma (5%) (Fig. 6A-D), when assessed after 1 month of in vivo growth. Moreover, apoptosis was prominent in the periductal fibroblasts of tissue recombinants prepared with $Egfr^{-/-}$ stroma (Fig. 6G), when assessed after 1 month of growth in the nude mouse host. Indeed, the condensed layer of periductal fibroblasts was entirely missing in the tissue recombinants prepared with both $Egfr^{-/-}$ stroma and $Egfr^{-/-}$ epithelium (Fig. 6H). Taken together, these results indicate that signaling through the EGFR must occur in the stroma surrounding the

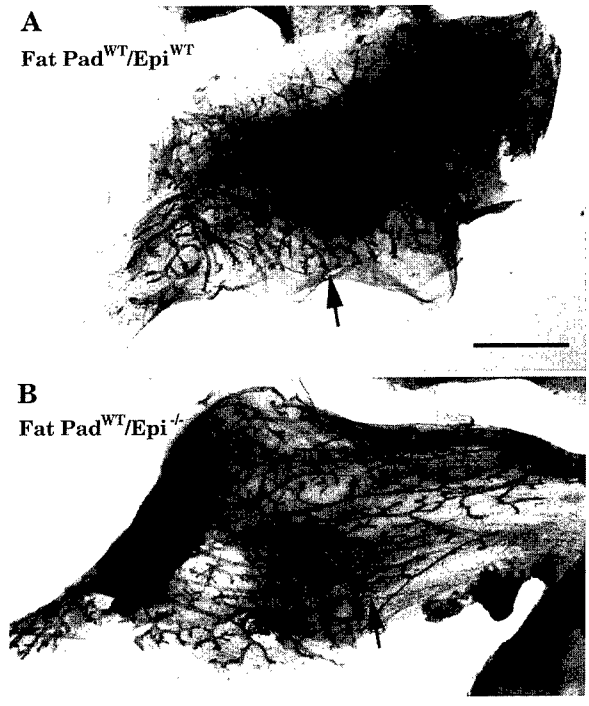

Fig. 3. Whole-mount preparations of transplants of either wild-type (A) or $Egfr^{-/-}$ (B) epithelium (arrows) into cleared fat pads of female nude mice hosts after two months of growth. The $Egfr^{-/-}$ epithelium undergoes ductal morphogenesis as does the wild-type epithelium, demonstrating that the $Egfr^{-/-}$ epithelium is competent, if given the correct developmental cues from normal stroma. Bar, 2 mm.

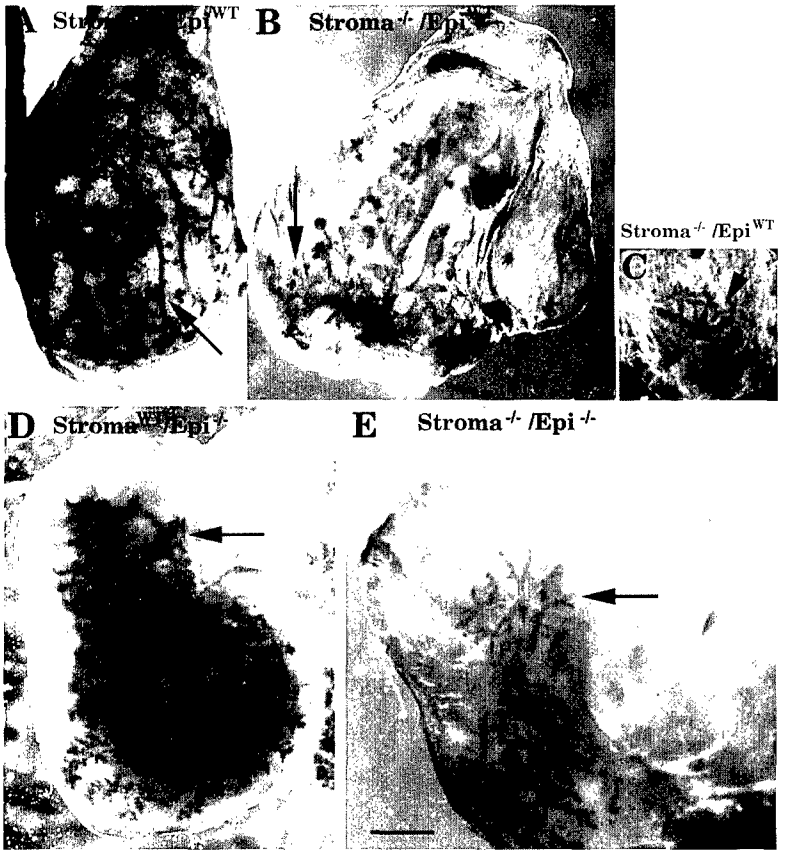

Fig. 4. Whole-mount preparations of tissue recombinants grown for one month under the kidney capsule of a nude mouse. The tissue recombinants with wild-type stroma, Stroma^{WT}/Epi^{WT} (A) and Stroma^{WT}/Epi$^{-/-}$ (D) have extensive ductal growth (arrows), while the tissue recombinants with *Egfr*$^{-/-}$ stroma, Stroma$^{-/-}$/Epi^{WT} (B,C) and Stroma$^{-/-}$/Epi$^{-/-}$ (E) have very little ductal growth (arrows). Two examples of tissue recombinants containing *Egfr*$^{-/-}$ stroma are shown (B,C). The amount of ductal growth seen in panel B is the most ever observed and is atypical. More commonly, the amount of ductal growth in the Stroma$^{-/-}$/Epi^{WT} tissue recombinants is meager as indicated in C. Bar, 1 mm.

epithelial ducts to induce normal ductal proliferation and morphogenesis in the mammary gland.

Lobulo-alveolar units develop in the *Egfr*$^{-/-}$ mammary gland

Lobulo-alveolar development takes place during pregnancy to prepare the mammary gland for lactation. During this process, terminal alveolar units differentiate from presumptive stem cells. Hormonal requirements for lobulo-alveolar development are entirely different from those required for ductal growth. Estradiol (E_2) stimulates ductal development during puberty, while progesterone and prolactin stimulate alveolar development during pregnancy (Daniel et al., 1987; Bocchinfuso and Korach, 1997; Haslam, 1988; Das and Vonderhaar, 1997; Lydon et al., 1995; Ormandy et al., 1997). The EGFR has been localized to the stroma surrounding the mammary ducts during puberty and in luminal epithelial cells during lactation (DiAugustine et al., 1997). Therefore, it was of interest to examine alveolar development in the *Egfr*$^{-/-}$ mammary gland. To accomplish this, neonatal mammary glands from wild-type or *Egfr*$^{-/-}$ mice were transplanted along with an adult pituitary under the renal capsule (n=8). The grafted pituitary secretes large amounts of prolactin, which stimulates alveolar development and β-casein expression in the mammary grafts (Adler, 1986; Cunha et al., 1992). Development of lobulo-alveolar units was nearly normal in *Egfr*$^{-/-}$ glands grown in pituitary-grafted hosts (Fig. 7B,D). β-casein gene expression was induced by the pituitary graft in both the *Egfr*$^{-/-}$ and WT glands as demonstrated by RT-PCR analysis (data not shown). These data indicate that the *Egfr*$^{-/-}$ mammary glands are competent to respond to other hormonal signals (e.g. prolactin, progesterone) to produce a lactational phenotype. However, alveolar development was not as dense in the *Egfr*$^{-/-}$ glands as in the wild-type glands (Fig. 7A-D),

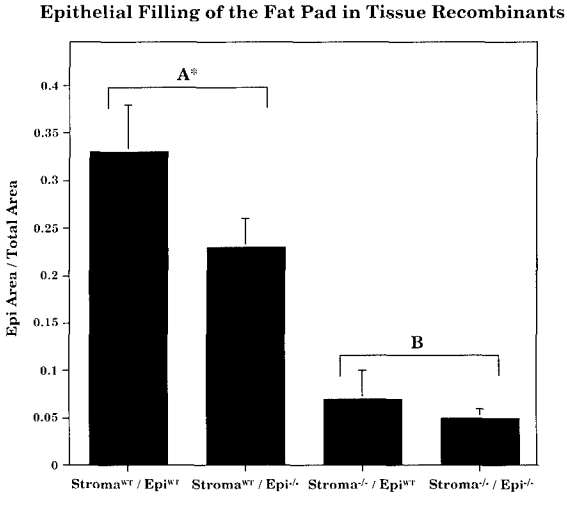

Fig. 5. Percentage of epithelial filling of the fat pad in tissue recombinants. The tissue recombinants that contained wild-type stroma (A) filled the pad pad to a greater extent ($P \leq 0.02$) than those prepared with *Egfr*$^{-/-}$ stroma (B) (n=3-5). However, there was not a significant difference between the tissue recombinants within group A (Stroma^{WT}/Epi^{WT} and Stroma^{WT}/Epi$^{-/-}$), or within group B (Stroma$^{-/-}$/Epi^{WT} and Stroma$^{-/-}$/Epi$^{-/-}$).

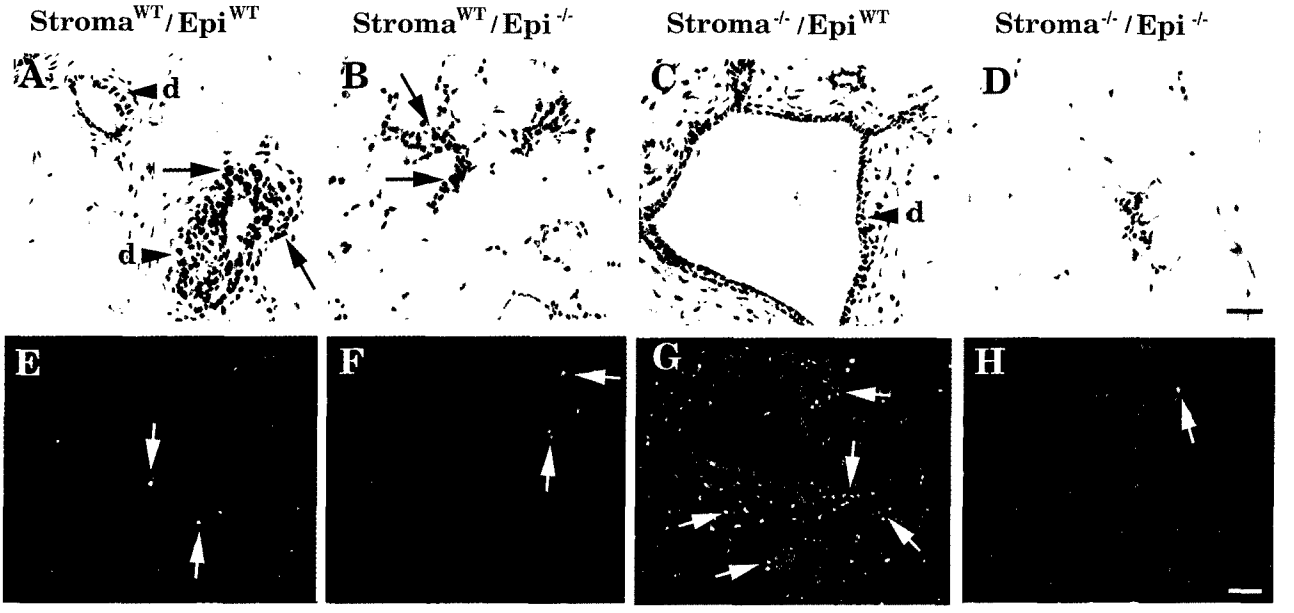

Fig. 6. Cell proliferation and apoptosis in tissue recombinants. (A-D) The proliferating cells were determined by BrdU incorporation and are stained brown (arrows). The tissue recombinants with wild-type stroma (A,B) have a considerable amount of proliferating cells in both the stroma and the ductal epithelial cells. The tissue recombinants with *Egfr*$^{-/-}$ stroma (C,D) have little to no proliferation. The large distended lumen of the epithelial duct (d) is apparent in the tissue recombinant with *Egfr*$^{-/-}$stroma (C) as compared to the ducts with WT stroma (A,B). (E-H) Apoptosis in tissue recombinants. Cells undergoing apoptosis are labeled with fluorescein (arrows). Apoptosis in the tissue recombinants with wild-type stroma (E,F) is seen mainly in the ductal epithelial cells. In the tissue recombinants with *Egfr*$^{-/-}$ stroma/wild-type epithelium (G) there is massive apoptosis in the periductal stroma, in addition to some apoptosis in the ductal epithelial cells. In the *Egfr*$^{-/-}$/*Egfr*$^{-/-}$ tissue recombinants (H), there is little apoptosis because there is only residual ductal formation at the end of the grafting period. Bar, (A-D) 50 μm, (E-H) 100 μm.

owing to the underlying defect in ductal development in the *Egfr*$^{-/-}$ glands (Fig. 7D). Both types of glands had similar numbers of proliferating (12%) (Fig. 7E,F) and apoptotic (2-4%) cells (Fig. 7G,H) when grown in pituitary-grafted hosts. Alveolar development was also stimulated by a pituitary graft when either *Egfr*$^{-/-}$ or wild-type epithelium was transplanted into wild-type cleared fat pads (data not shown).

DISCUSSION

Signaling through the EGFR is necessary for ductal morphogenesis

Our study using the grafting of neonatal mammary glands has demonstrated that signaling through the EGFR pathway is essential for mammary ductal development. The EGFR is normally present in the stromal fibroblasts that separate the ducts from the fatty stroma (DiAugustine et al., 1997; Cunha and Hom, 1996). Impaired ductal growth in *Egfr*$^{-/-}$ mammary glands was associated with a marked reduction in the density of periductal fibroblasts. Thus, signaling through the EGFR may promote fibroblast survival, which in turn induces ductal epithelial cell proliferation. The reduction in periductal fibroblasts in the *Egfr*$^{-/-}$ grafts due to reduced proliferation and increased apoptosis results in a profound impairment in ductal morphogenesis.

Six different ligands (EGF, TGF-α, AR, BTC, HB-EGF and epiregulin) have been reported to signal through the EGFR. EGFR ligands are expressed in multiple cell types of the mammary gland (DiAugustine et al., 1997; Snedeker et al., 1992) and, apparently, have compensatory functions, because mice homozygous null for TGF-α display no overt mammary phenotype (Luetteke et al., 1993). However, overexpression of TGF-α in transgenic mice results in mammary hyperplasia, with an increased incidence and decreased latency of mammary tumorigenesis (Matsui et al., 1990; Jhappan et al., 1990; Sandgren et al., 1990; Coffey et al., 1994; Halter et al., 1992). Although the contributions of the various EGFR ligands in promoting ductal development is still under consideration, it is clear that signaling through the EGFR is absolutely necessary to optimally stimulate ductal growth and branching. Signaling through the EGFR has been shown to be important for the establishment of branching morphogenesis of the trachea in *Drosophila* (Wappner et al., 1997). Our results demonstrate not only that signaling through the EGFR is required for mammary ductal growth and development, but that other ErbB family members known to be present in the mammary gland (Pinkas-Kramarski et al., 1997) cannot compensate for the lack of the EGFR to promote ductal morphogenesis.

Other growth factors produced by mammary stroma can stimulate ductal growth. Hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) are stromal factors that stimulate ductal growth and development (Yang et al., 1995; Niranjan et al., 1995; Soriano et al., 1995; Imagawa et al., 1994; Yi et al., 1994; Ulich et al., 1994). Transgenic mice that express KGF under the control of the mouse mammary tumor virus (MMTV) promoter develop mammary tumors (Kitsberg

and Leder 1996). However, in the absence of *Egfr*, endogenous HGF and KGF cannot compensate to promote ductal morphogenesis, although it is unknown whether these growth factors are expressed normally in the *Egfr*$^{-/-}$ mouse mammary gland.

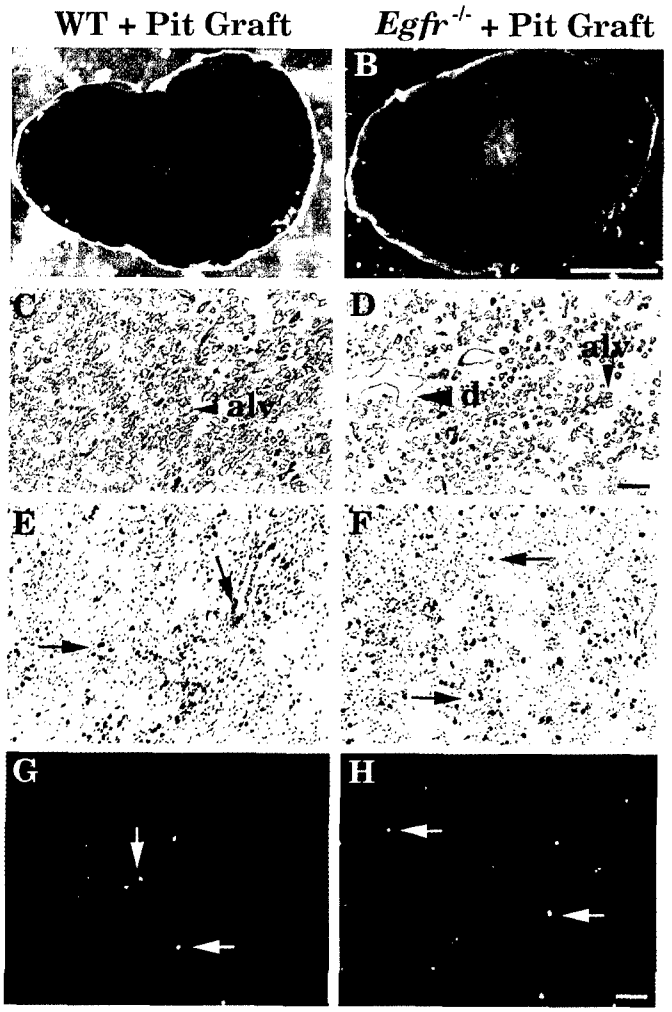

Fig. 7. Whole-mount preparations, cell morphology, proliferation and apoptosis in intact neonatal mammary glands grown in pituitary grafted female nude mouse hosts for one month. (A,B) Whole-mount preparations of mammary glands grafted with a pituitary gland. Alveolar development produced in response to the grafted pituitary is not compromised in the *Egfr*$^{-/-}$ mammary gland (B), although the alveoli do not penetrate throughout the entire fat pad as seen with the wild-type graft (A). (C,D) Cell morphology of mammary glands grafted with a pituitary gland. In response to a pituitary graft, alveolar development (alv) occurs in both the *Egfr*$^{-/-}$ (D) and wild-type (C) glands. The alveolar development is not as dense in the *Egfr*$^{-/-}$ gland as in the wild-type gland due to the underlying defect in ductal morphogenesis. The large distended lumen seen in the *Egfr*$^{-/-}$ gland is indicated (d). (E,F) Cell proliferation in mammary glands grafted with a pituitary gland as determined by BrdU incorporation. The proliferating cells are stained brown (arrows). Both wild-type (E) and *Egfr*$^{-/-}$ (F) glands have a large percentage of proliferating cells during alveolar development. (G,H) Apoptosis in mammary glands grafted with a pituitary gland. Cells undergoing apoptosis are labeled with fluorescein (arrows). Wild-type (G) and *Egfr*$^{-/-}$ (H) glands have similar levels of apoptosis during alveolar development. Bar, (A,B) 2 mm, (C,D) 200 μm, (E-H) 100 μm.

Other molecules that may mediate cell-cell interactions in the mammary gland include the Wnt proteins (Gavin and McMahon, 1992; Bühler et al., 1993; Weber-Hall et al., 1994), homeobox-containing genes such as *Msx-1*, *Msx-2*, *Bmp-2* and *Bmp-4* (Friedmann and Daniel, 1996: Phippard et al., 1996; Pavlova et al., 1994), parathyroid hormone-related protein (PTHrP) (Wysolmerski et al., 1995; Wysolmerski et al., 1998) and the matrix metalloproteinases (Sympson et al., 1994; Wiesen and Werb, 1996; Werb et al., 1996). However, while many factors may influence mammary growth and development, our data indicate that the EGFR is an essential component of the stromal signaling cascade that controls growth and ductal branching morphogenesis in the mammary gland.

Lobulo-alveolar development is not compromised in the *Egfr*$^{-/-}$ mammary gland

In contrast to the lack of ductal development, alveolar development occurred in grafts of both *Egfr*$^{-/-}$ and wild-type mammary glands in response to prolactin produced by the pituitary graft. Although the alveoli were morphologically normal in grafts of *Egfr*$^{-/-}$ mammary glands, these structures did not penetrate entirely throughout the fat pad of the *Egfr*$^{-/-}$ mammary glands compared to the wild-type glands, presumably due to the underlying defect in ductal development. This defect was not intrinsic to the epithelium, because *Egfr*$^{-/-}$ epithelium transplanted into wild-type cleared pads showed normal ductal development and equivalent alveolar development in response to a pituitary graft. Therefore, signaling through the EGFR is dispensable for alveolar development, just as it is indispensable for ductal development. Similarly, ductal development is impaired, while lobulo-alveolar development remains normal in transgenic mice expressing a dominant negative, truncated version of EGFR in the mammary gland under the control of the MMTV-long terminal repeat (Xie et al., 1997). Interestingly, the *waved-2* (*Egfr*$^{wa-2/wa-2}$) mouse, which has a point mutation in the EGFR kinase domain resulting in diminished signaling, displays impaired lobulo-alveolar development and decreased lactation (Fowler et al., 1995). Whether this is due to an underlying defect in ductal development or a result of abnormal development of other endocrine organs, which are required to maintain the hormonal milieu necessary for lactation has not been determined. However, the viability of these mice and the greater degree of their mammary development suggests that the functional EGFR probably is the heterodimer with ErbB2.

Heregulin (neuregulin) is produced by mammary stromal cells during the lobulo-alveolar development during pregnancy, and binds to ErbB3 and ErbB4 (Carraway and Cantley, 1994). Transgenic mice that express heregulin in the mammary gland under the control of the MMTV promoter form adenocarcinomas, and ErbB3 was the only receptor phosphorylated (Krane and Leder, 1996). In culture, heregulin stimulates alveolar development (Yang et al., 1995). Thus, heregulin may compensate for the lack of EGFR in the *Egfr*$^{-/-}$ mammary gland by directly binding to and signaling through ErbB3 and ErbB4.

Prolactin and progesterone are necessary for lobulo-alveolar development (Haslam, 1988; Das and Vonderhaar, 1997). Alveolar development is abolished in mice lacking either the progesterone receptor (Lydon et al., 1995) or the prolactin

receptor (Ormandy et al., 1997). While ductal growth is unaffected in mice lacking the progesterone receptor, ductal growth is impaired in virgin mice lacking the prolactin receptor (Ormandy et al., 1997). Although ductal and alveolar development in the mammary gland share key regulatory molecules that transduce systemic hormone action such as the steroid receptor coactivator-1 (SRC-1) (Xu et al., 1998), we can conclude that ductal and alveolar development are also controlled by distinct pathways at the local level by growth factors via stromal-epithelial interactions.

EGFR is a mediator of stromal-epithelial interactions during mammary ductal morphogenesis

Understanding the nature of stromal-epithelial interactions that regulate mammary growth and function is crucial to understanding ductal morphogenesis and alveolar development. Members of the inhibin/activin family may be one component of the regulatory molecules that locally influence ductal morphogenesis and alveolar development via stromal-epithelial interactions (Hennighausen and Robinson, 1998). Interestingly, mice that lack the inhibin βb subunit (*Inhbb*$^{-/-}$) needed for activin and inhibin signaling have a defect in both ductal and alveolar development. Transplantation of *Inhbb*$^{-/-}$ epithelium into wild-type cleared fat pads results in normal development suggesting that only stromal inhibin bb is necessary (Robinson and Hennighausen, 1997). Although inhibin βb is a stromally derived factor that controls epithelial growth, its action appears not to be restricted to ductal morphogenesis.

Ductal growth of the mammary gland is estrogen-dependent and is profoundly impaired in mice with a null mutation in the estrogen receptor-α (*Estra*$^{-/-}$, ERKO) (Bocchinfuso and Korach, 1997). Tissue recombinant studies using the fat pads and mammary gland epithelia from *Estra*$^{-/-}$ (ERKO) and wild-type mice demonstrate that estrogen stimulates mammary ductal epithelial growth via a paracrine mechanism, acting through the stromal estrogen receptor. The epithelial estrogen receptor is neither necessary nor sufficient for ductal development (Cunha et al., 1997). EGFR signaling is thought to be a downstream effector of estrogen action in several target organs (Mukku and Stancel, 1985a,b; DiAugustine et al., 1988; Sakai et al., 1994; Nelson et al., 1994). Mammary gland epithelium expresses EGFR, and EGF and TGF-α are mitogens for mammary gland epithelial cells (DiAugustine et al., 1997). Despite this, our data clearly show that signaling through the EGFR is not essential in the epithelial component of the mammary gland in vivo. Instead, the EGFR is absolutely necessary for the stromal component, the fat pad, to induce estrogen-dependent ductal growth and branching morphogenesis as shown by the tissue recombination studies. These results suggest that, under estrogenic conditions, which stimulate the pubertal mammary gland, the stroma responds to estrogen action through an EGFR-mediated signaling event that is required for stimulation of epithelial growth and development. In contrast, the epithelial EGFR is neither necessary nor sufficient.

Transgenic mouse models and the development of tissue recombinant technology have allowed identification of several stromally derived regulatory molecules. Whether these factors are present in a single pathway or in parallel pathways awaits further epistatic experiments to order these components.

Mammary tumorigenesis – a disease of altered stromal-epithelial interactions

While most mammary tumors are derived from ductal epithelium, the surrounding stroma plays a crucial supporting role via reciprocal cell-cell interactions. The stromally derived signals that influence tumor formation and growth could be via growth factors, homeobox-containing genes that specify cell fate, changes in adhesion molecules, production of metalloproteinases, changes in the extracellular matrix, or regulation of cell proliferation and apoptosis (Krane and Leder, 1996; Kitsberg and Leder, 1996; Lundy et al., 1991; Friedmann and Daniel, 1996; Werb et al., 1996; Wiesen and Werb, 1996). The overexpression of ErbB family members and their ligands is frequently seen in cancers of the breast. This overexpression is correlated with poor prognosis, as these growth factors and receptors appear to be expressed to a greater degree in malignant than in normal breast tissue (Sainsbury et al., 1985; Fitzpatrick et al., 1984; Klijn et al., 1992). The majority of breast cancers that overexpress growth factors, also overexpress the EGFR, which may set up an autocrine loop to escape hormone dependence (Lundy et al., 1991; Umekita et al., 1992). In fact, the overexpression of the EGFR and the ErbB2 receptor are associated with progression to hormone-independence in human breast cancer (Sainsbury et al., 1985; Fitzpatrick et al., 1984; Klijn et al., 1992). It would be interesting to determine whether the stroma loses its normal capacity to regulate epithelial growth via reciprocal stromal-epithelial interaction when growth factors and their receptors are expressed in the epithelial tumors. If the mechanism through which stromal EGFR signaling regulates epithelial growth and development were identified, this could lead to strategies for intervention in cancer.

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Uterine and Vaginal Organ Growth Requires Epidermal Growth Factor Receptor Signaling from Stroma*

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ABSTRACT

Estrogens are crucial for growth and function of the female genital tract. Recently, we showed that induction of uterine epithelial proliferation by estradiol is a paracrine event requiring an estrogen receptor-positive stroma. Growth factors [such as EGF (epidermal growth factor) ligands] are likely paracrine mediators, which may directly or indirectly regulate epithelial proliferation in estrogen target organs via cell-cell interactions. In this report, we used mice with a null mutation in their EGF receptor (EGFR) to examine the role of EGFR signaling in growth of the uterus and vagina and in estrogen-induced uterine and vaginal epithelial proliferation. When WT and EGFR-knockout (EGFR-KO) uteri and vaginae were grown as renal capsule grafts in nude mice, growth of uterine and vaginal grafts of EGFR-KO mice was reduced, compared with their WT counterparts. Grafts of both EGFR-KO uteri and vaginae were about one third smaller (wet weight) than their corresponding WT organs, even though differentiation of both epithelium and mesenchyme were normal in both cases. Both wild-type and EGFR-KO vaginal grafts contained within their lumina alternating layers of cornified and mucified epithelial cell layers, indicating cyclic alteration of epithelial differentiation. In response to estradiol treatment, stromal cell labeling index (LI), as assessed by incorporation of ^{3}H-thymidine, was severely depressed in EGFR-KO uterine and vaginal grafts *vs.* stromal cell LI in WT uterine and vaginal grafts. Unexpectedly, epithelium of both EGFR-KO and wild-type grafts responded comparably to estradiol with a marked elevation (~7-fold overall) of epithelial LI in response to estradiol in uterine and vaginal epithelia. These data supported the hypothesis that overall uterine and vaginal organ growth, in response to estrogen, required EGFR signaling for DNA synthesis in the fibromuscular stroma, whereas EGFR signaling was not essential for estrogen-induced epithelial growth in the uterus and vagina. (*Endocrinology* **139: 913–921, 1998**)

THE EPIDERMAL growth factor (EGF) family of ligands, which includes EGF, TGFα, heparin-binding EGF (HB-EGF), amphiregulin, heregulin, epiregulin, and several other molecules (1, 2), is implicated in uterine and vaginal development, particularly as mediators of estrogen action. The possible role of the EGF family in estrogenic effects in the female genital tract is supported by many studies. Estrogens have effects upon both EGF and EGF receptors in the uterus (3, 4) and vagina (5) *in vivo*. Estradiol injected into immature female rats elicited a 3-fold increase in specific, high-affinity, saturable binding of ^{125}I-EGF to uterine membranes (6, 7). After estrogen injection, uterine EGFR binding increased between 6–12 h, remained elevated at 18 h, and declined thereafter. This increase in EGFR-binding was blocked by both cycloheximide and actinomycin D and was specifically induced by estrogens but not by nonestrogenic hormones (6). A corresponding study, using ovariectomized rats, showed a 2- to 3-fold elevation of uterine immunodetectable EGFR 18 h after injection of estradiol (8). Uterine EGFR transcripts were elevated in ovariectomized rats (9) within 3 h of estradiol injection, remained elevated at 6 h after estradiol injection, and then declined thereafter. Estrogenic effects were blocked by actinomycin D but not by puromycin. Nonestrogenic hormones did not mimic the estrogen-mediated increase in EGFR messenger RNA (mRNA) levels. However, for all of the above studies, it was unclear whether the effect of estradiol on uterine EGFR reflected changes in the epithelial, stromal, or myometrial compartments. Nonetheless, these studies suggested that estrogen-dependent growth of the uterus and vagina were mediated via EGF ligands acting through the EGFR.

Paracrine models of estrogen action in the mouse uterus and vagina were considered for many years because estrogen receptors were expressed in the epithelium, stroma, and myometrial cells (10–12). Thus, it was possible that estradiol elicited epithelial effects by acting directly upon the epithelium via epithelial estrogen receptors or via estrogen receptors in stromal cells, which in turn, stimulated epithelial proliferation in a paracrine fashion. It was also unknown whether stromal proliferation was regulated by direct or paracrine action of estradiol. It was initially assumed that the myriad effects of estradiol on epithelium were mediated directly through epithelial estrogen receptors. However, analysis of estrogen receptor expression and estradiol responsiveness in the neonatal mouse uterus indicated that this was not correct. Using neonatal Balb/c mice, Cunha *et al.* (13) demonstrated, with steroid autoradiography, that estrogen receptors were undetectable in uterine epithelium (UtE) but were present in uterine mesenchyme (UtM). Despite the apparent lack of uterine epithelial estrogen receptors in the neonatal mouse, injection of diethylstilbestrol (DES) caused a doubling in the rate of UtE proliferation (14). One explanation for these results could be that DES induced the ex-

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pression of epithelial estrogen receptors that, in turn, mediated the mitogenic effects of DES on the epithelium. However, Bigsby and Cunha (14) showed that estrogen receptors remained undetectable in the UtE, even after DES stimulation. These results suggested that the mitogenic effects of DES on neonatal UtE could be elicited via paracrine influences from estrogen receptor-positive mesenchymal cells. More recent immunohistochemical studies were consistent with this interpretation (15–18).

To determine roles of epithelial *vs.* stromal estrogen receptors in uterine epithelial proliferation, a transgenic estrogen receptor knockout (ERKO) mouse (19) was used to produce uterine tissue recombinants in which epithelium (UtE), stroma (UtS), or both were devoid of functional estrogen receptors. In tissue recombinants prepared with wild-type (WT) uterine stroma (WT-UtS + WT-UtE and WT-UtS + ERKO-UtE), epithelial labeling index (LI) was increased severalfold by estradiol over oil-treated controls (20). In contrast, in tissue recombinants prepared with ERKO uterine stroma (ERKO-UtS + ERKO-UtE and ERKO-UtS + WT-UtE), epithelial LI was low and similar in estradiol- *vs.* oil-treated specimens. These data clearly demonstrated that estradiol induction of uterine epithelial proliferation was a paracrine event requiring an estrogen receptor-positive stroma. Moreover, epithelial estrogen receptors were neither necessary nor sufficient for estradiol-induced epithelial proliferation. These findings suggested the existence of paracrine mediators of stromal origin, which directly or indirectly regulated epithelial proliferation in estrogen target organs. Growth factors (such as the EGF family of ligands) are likely candidates of such putative paracrine mediators.

To examine the role of EGFR signaling in estrogen-dependent growth of the uterus and vagina, we used a transgenic mouse deficient in EGFR signaling (21), which would be predicted to exhibit impaired uterine and vaginal growth and impaired estrogenic response. These EGFR-KO mice showed growth retardation and epithelial dysfunction, which resulted in gastrointestinal and lung abnormalities resembling human diseases associated with premature birth. EGFR-KO homozygotes displayed epithelial immaturity and multiorgan failure, whereas the heterozygotes developed normally. Some homozygous EGFR-KO embryos died prenatally, but many survived into the early neonatal period before succumbing. Nevertheless, organ rudiments could be rescued from EGFR-KO neonates by grafting them into athymic nude mouse hosts so that estrogenic response could be examined. Using these methods, we investigated the complex interplay between estrogen action, paracrine stromal-epithelial interactions, and EGFR signaling in growth of the uterus and vagina.

Materials and Methods

Animals. EGFR-KO mice (21) were bred at the University of California, San Francisco. EGFR-KO mice were typed at birth by their so-called open eye phenotype. Normal female Balb/c mice were obtained from the Cancer Research Laboratory, University of California, Berkeley, CA. Intact female athymic nude mice were purchased from Harlan (Indianapolis, IN). For this report, 50 female EGFR-KO newborns, 48 female WT littermates, and 100 nude mice were used. All animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all procedures described here were approved by the University of California, San Francisco, animal care and usage committees. Mice were maintained under controlled temperature and lighting conditions during the experiment and were given food and water *ad libitum*.

Microdissection and tissue recombinations. Female EGFR-KO mice, normal littermates, and normal female Balb/c mice were killed at 0–3 days postnatal, and entire genital tracts were removed by dissection. For whole-organ grafts, uteri and vaginae were trimmed as indicated (Fig. 1) and grafted under the renal capsule of female athymic nude mice.

Grafting, ovariectomy, and graft harvest. Whole-organ rudiments from EGFR-KO and WT animals were transplanted beneath renal capsules of female athymic nude mouse hosts. After 1 month of growth beneath the renal capsule, the nude mouse hosts were ovariectomized; 7 days later, the nude mouse hosts were given one injection (ip) of either 125 ng estradiol (Steroids, Wilton, NH) or corn oil (control). Eighteen hours later, the hosts were injected ip with 1.5 μCi/g BW of ^{3}H-thymidine (specific activity = 84 Ci/mmol) (Amersham, Arlington Heights, IL) and killed 2 h later. The grafts were harvested and fixed in 4% formalin. Harvested grafts were imaged using a color Lumina camera (Leaf System, South Brough, MA) to document overall size and gross morphology. Wet weights were determined for all grafts.

Histology. The grafts were harvested, fixed in 4% buffered formaldehyde, embedded in paraffin, and sectioned at 6 μm. For histological analysis, specimens were stained with hematoxylin and eosin.

Autoradiography. For analysis of epithelial and stromal LI, paraffin sections of the specimens were mounted on glass slides, dipped in NTB-II

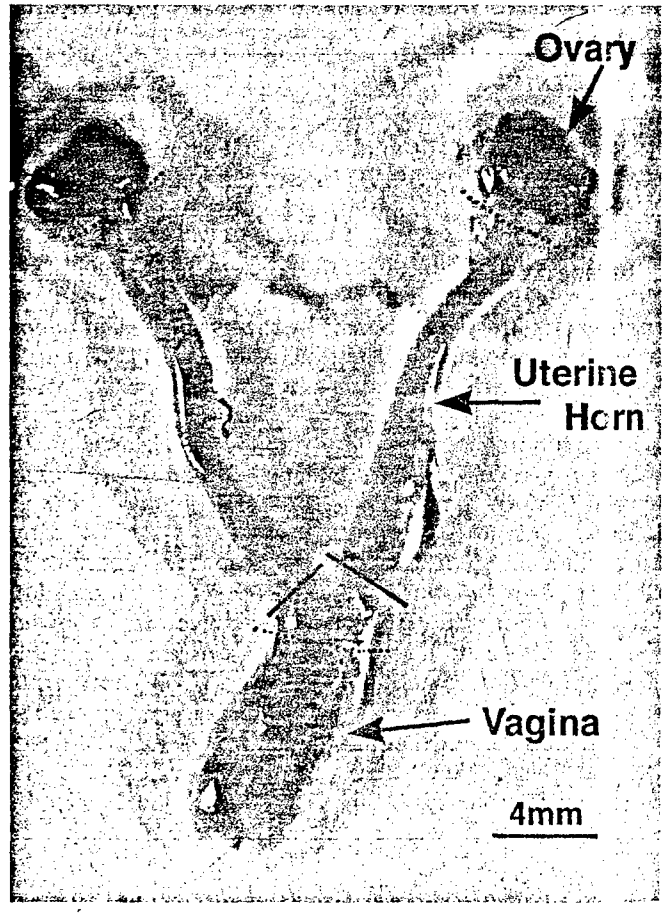


FIG. 1. Female EGFR-KO and Balb/c mice were killed at 0–3 days postnatal, and the entire genital tract was removed by dissection. Whole-organ pieces, for grafting, were trimmed as indicated by the *lines*. The ovaries, uterine horns, and vagina are indicated. The *bar* equals 4 mm.

photographic emulsion (Kodak, Rochester, NY), and processed autoradiographically via standard protocols (22).

LI. Epithelial LI and stromal LI with ^{3}H-thymidine was determined as the percentage of labeled epithelial or stromal cells in the total population of epithelial or stromal cells, as described previously (22). Individual histological sections to be scored were selected randomly, and for a given section, only regions of appropriate section orientation were scored in which the plane of section was roughly perpendicular to the plane of the epithelial basement membrane. Areas of poor section quality, tangential, or oblique orientation were excluded. For each type of graft, a minimum of 300 cells were scored per individual specimen for 3–6 replicate specimens.

Statistics. Values were expressed as the mean ± SEM of at least six different experiments. Differences among means were estimated using a Student's unpaired t test and ANOVA. Differences were considered significant at $P < 0.05$.

Results

Growth, but not differentiation, was impaired in grafted uteri and vaginae from EGFR-KO mice. In general, grafts of EGFR-KO uteri and vaginae grown for 1 month in intact female hosts were smaller than their WT littermates, as judged by wet weight at harvest and overall size of the grafts. Grafted WT uteri from estradiol-treated hosts (0.299 ± 0.114 g, n = 3) were 1.6 times larger than uteri derived from EGFR-KO mice (0.187 ± 0.065 g, n = 3). Each set of individual experiments was performed with grafts from EGFR-KO mice of slightly different ages (0–3 days postnatal). The age of the graft at transplantation affected the graft's weight gain. Thus, overall size and weight of grafts varied from experiment to experiment because of differences in specimen age at the time of grafting. However within individual experiments, grafts of WT uteri always attained a larger size and weight than grafts of EGFR-KO uteri. Grafts of undifferentiated neonatal uterine rudiments from WT donors developed normally. Both luminal epithelium and uterine glands developed from the undifferentiated Müllerian duct epithelium of the neonatal uterus. The undifferentiated UtM of the grafted neonatal uterus differentiated into endometrial stroma and myometrium (Fig. 2a). Although grafts of EGFR-KO uteri were somewhat smaller than WT grafts after 1 month of growth, development of both epithelium and mesenchyme was normal and was equivalent to that of grafts of WT uteri (Fig. 2, a and b).

Grafted WT vaginae from estradiol-treated hosts (0.226 ± 0.030 g, n = 3) were 1.5 times larger than corresponding organs derived from EGFR-KO mice (0.149 ± 0.029 g, n = 3). As mentioned above, each individual experiment was performed with grafts from EGFR-KO mice of slightly different ages, which led to some variability in overall graft size and weight from experiment to experiment. However, within a given experiment, vaginal growth was consistently elevated in WT *vs.* EGFR-KO grafts. As was the case for uteri, grafts of neonatal vaginae of WT and KO mice developed normally and formed a highly differentiated vaginal epithelium whose differentiation varied with the stage of the estrous cycle of the host at the time of death. The lumina of the vaginal grafts were filled with alternating layers of cornified and mucified epithelial cells in both WT and EGFR-KO vaginal grafts, indicating cyclical alteration in epithelial differentiation (Fig. 2, c and d).

Stromal cells in grafted uteri and vaginae from EGFR-KO mice had an impaired proliferative response to estradiol. We found that overall growth (wet weight) was reduced in grafts of EGFR-KO *vs.* WT uteri and vaginae. To explain this difference in size of EGFR-KO uterine and vaginal grafts, we determined cell proliferation by analyzing incorporation of ^{3}H-thymidine and stromal cell LI's in grafts of intact WT and EGFR-KO uteri. LI for uterine stromal cells of EGFR-KO uteri in response to estradiol was indistinguishable from that of oil-treated controls (Fig. 3A). This contrasts with an increase in stromal cell LI of WT uterine grafts treated with estradiol, which was 4.6 times higher than that of WT uteri treated with oil and 2.3 times higher than that of its EGFR-KO counterpart. Similarly, in estradiol-treated EGFR-KO vaginal grafts, stromal cell LI was indistinguishable from that of its oil-treated counterpart (Fig. 3B). In contrast, in estradiol-treated WT vaginal grafts, stromal cell LI was 19.5 times higher than that of its estradiol-treated EGFR-KO counterpart and 6.4 times higher than its WT oil-treated counterpart. These data indicated that stromal response to estradiol was markedly impaired in EGFR-KO mice.

Estradiol induces epithelial growth in grafted uteri and vaginae of EGFR-KO mice. The proliferative response to estradiol in grafts of WT and EGFR-KO uteri and vaginae was assessed by determining LI. As shown in Fig. 4A, UtE of both EGFR-KO and WT uterine grafts responded comparably to estradiol by incorporation of ^{3}H-thymidine. Epithelial LI in WT uterine grafts increased approximately 4-fold in response to estradiol, relative to WT uterine grafts treated with oil. Epithelial LI in EGFR-KO uterine grafts treated with estradiol increased approximately 9 times, relative to that of EGFR-KO uterine grafts treated with oil. Epithelial LI's for WT and EGFR-KO uterine grafts treated with oil were not significantly different from each other at $P < 0.05$.

As was the case for the uterus, both EGFR-KO and WT vaginal grafts exhibited a marked epithelial proliferative response to estradiol (Fig. 4B). Epithelial LI's in WT vaginal grafts treated with estradiol were elevated approximately 3-fold, relative to epithelial LI of WT vaginal grafts treated with oil. Similarly, EGFR-KO vaginal grafts treated with estradiol exhibited an increase in epithelial LI approximately 11 times higher than that of EGFR-KO vaginal grafts treated with oil. Epithelial LI's for WT and EGFR-KO vaginal grafts treated with oil were not significantly different from each other at $P < 0.05$.

Discussion

Growth of organs containing an epithelial parenchyma was generally impaired in EGFR-KO mice, which led to a spectrum of deleterious lesions, principally in the lung and gastrointestinal tract, that compromised postnatal survival. Though EGFR-KO mice usually died in the neonatal period, undifferentiated uteri and vaginae could be isolated from neonatal EGFR-KO mice and grafted into athymic nude hosts. In this way, it was possible to grow these undifferentiated neonatal organs sufficiently long in an endocrinologically normal environment to achieve full maturation and

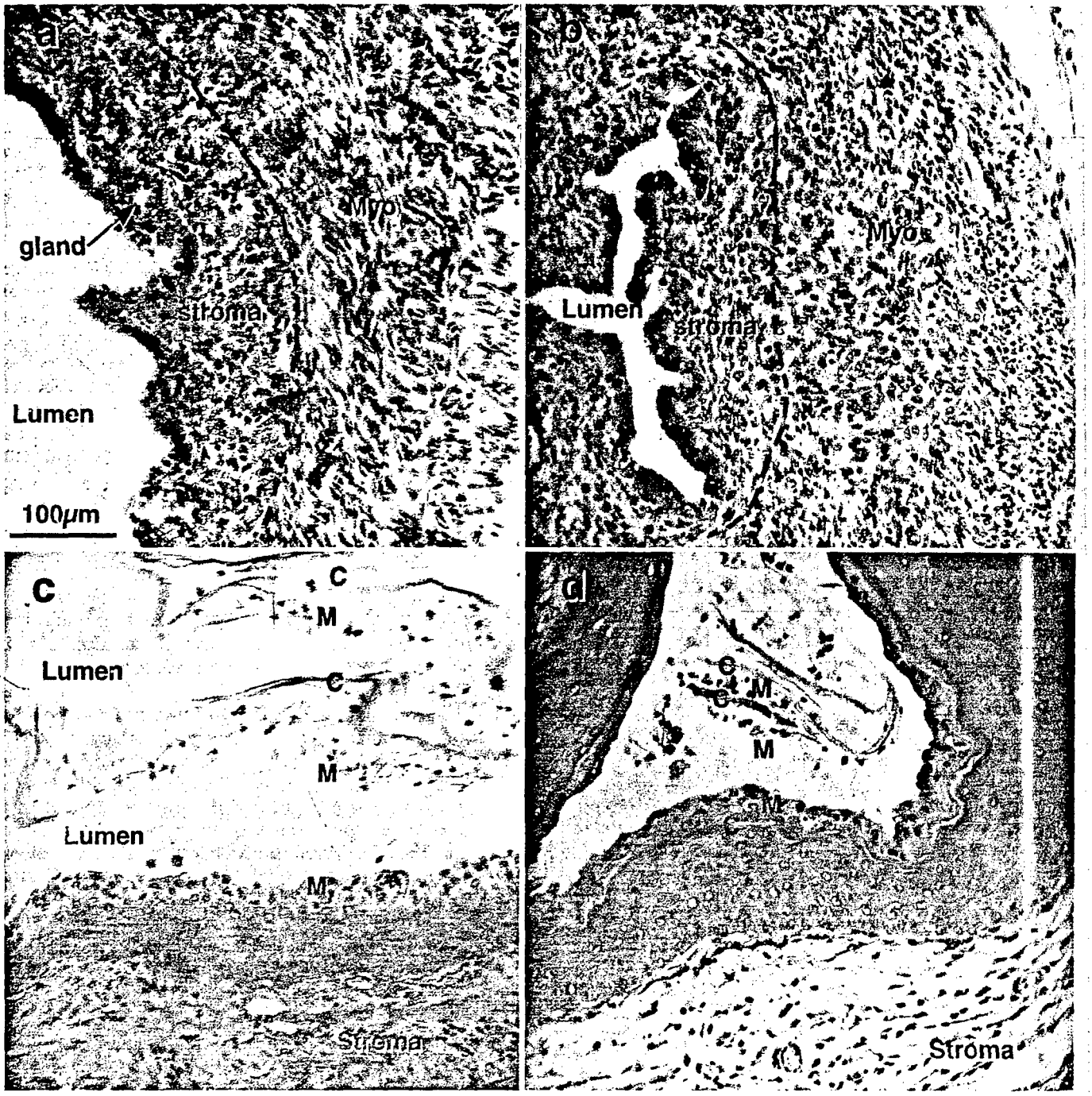
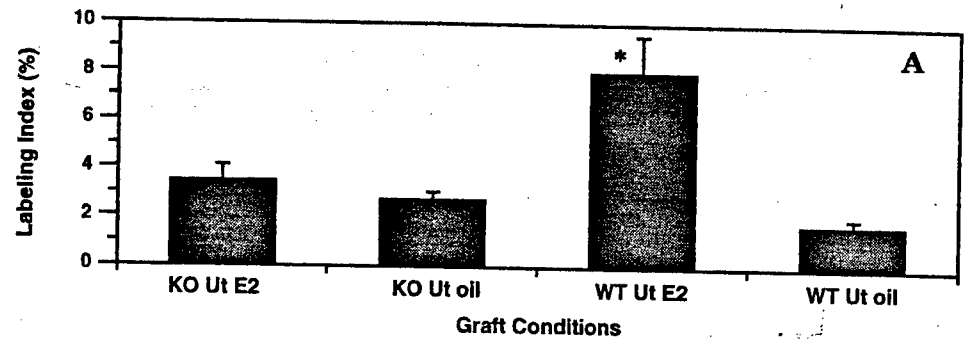


FIG. 2. Hematoxylin and eosin-stained sections of WT and EGFR-KO uterine and vaginal grafts. Panel a, WT uterus; panel b, EGFR-KO uterus; panel c, WT vagina; panel d, EGFR-KO vagina. For the uteri (a and b), the *dotted lines* delineate the stroma from the myometrium (Myo). In panel a, a uterine gland is indicated. For the vaginae (c and d), alternating cornified (C) and mucified (M) layers are indicated in the lumen. The *bar* equals 100 μm.

thereby enable the study of the estrogenic response in EGFR-KO uterine and vaginal grafts. Using these methods, we investigated the interplay between estrogen action and EGFR signaling. As shown in Fig. 2, histological analysis of uterine and vaginal grafts indicated that their epithelia grew and differentiated normally when grafted into intact female nude mouse hosts. Our group routinely grafted embryonic and neonatal organs from both male and female rodents. These renal capsule grafts displayed normal growth, morphogenesis, and differentiation. Moreover, markers of adult function were expressed by grafts of embryonic or neonatal organ rudiments usually by 3–4 weeks after grafting. For example, lactotransferrin was expressed by neonatal uterine grafts or homotypic neonatal uterine tissue recombinants (UtM + UtE) grown for 4 weeks in adult female hosts (20). For this reason, it was important to recognize that our ex-



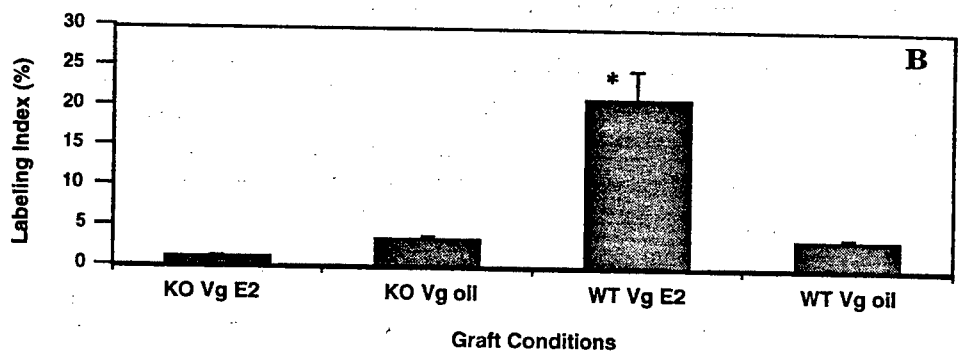
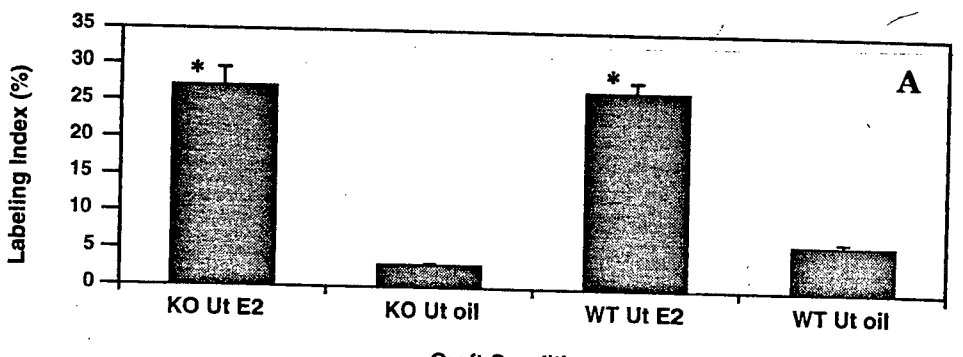


FIG. 3. Stromal cell LIs in WT (WT) and EGFR-KO (KO) uterine and vaginal grafts. Stromal cell LIs with ^{3}H-thymidine were determined, as the percentage of labeled stromal cells per total stromal cells counted. A, WT and EGFR-KO uterine stroma; B, WT and EGFR-KO vaginal stroma. Each *bar* represents the mean of at least six different experiments ± SEM. *, Statistical significance at $P < 0.05$; Ut, uterus; Vg, vagina; E2, estradiol.



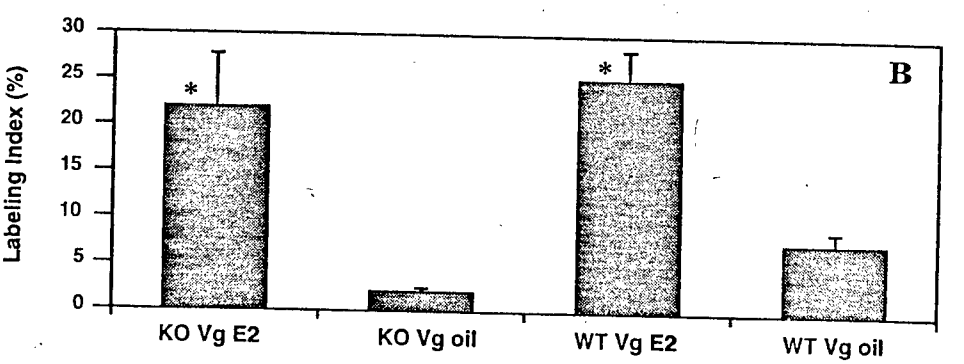


FIG. 4. Epithelial cell LIs in grafts of WT (WT) and EGFR-KO (KO) uteri and vaginae. Epithelial cell LI with ^{3}H-thymidine was determined, as the percentage of labeled epithelial cells per total epithelial cells counted. A, WT and EGFR-KO uterus; B, WT and EGFR-KO vagina. Each *bar* represents the mean of at least six different experiments ± SEM. *, Statistical significance at $P < 0.05$. Ut, Uterus; Vg, vagina; E2, estradiol.

perimental model did not relate to hormonal response of neonatal organs but instead, more appropriately, to postnatal organs at on advanced stage of functional differentiation comparable, in many ways, to adult organs.

The EGFR-KO mouse was described to undergo multiorgan failure (21), which led to its demise in the early neonatal period. Given the short life span of the EGFR-KO mouse, it was not possible to determine whether congenital abnor-

malities were reversible with time. Moreover, in the original description of the EGFR-KO mouse (21), only a limited number of organs were examined. Given this background it was striking that uterine and vaginal development was so normal in uterine and vaginal grafts from EGFR-KO neonatal mice. Perhaps the effects of EGFR-KO were variable in different organs. Alternatively, developmental abnormalities that existed during development of EGFR-KO uteri and vaginae were not observed at the end of our experiments because, given the extended period of growth in the nude mouse hosts, such abnormalities could be repaired through compensatory mechanisms. In any case, it should be emphasized that the embryonic and early neonatal development of the female Müllerian ducts and the urogenital sinus was normal in EGFR-KO mice. For this reason, the neonatal female genital tract of the EGFR-KO mouse was slightly smaller than, but otherwise indistinguishable from, the WT.

Many of the organs that were adversely affected in the EGFR-KO mouse were composed of epithelium and mesenchyme. In the present study, we found that the uterus and vagina of EGFR-KO mice exhibited a generalized growth deficit of 34–38%. Although EGFR signaling was absent simultaneously in both epithelium and mesenchyme in EGFR-KO mice, our data suggested that the lack of functional EGFR in uterine and vaginal stroma was the key event accounting for overall organ hypoplasia in the female genital tract and impaired overall growth of EGFR-KO *vs.* WT uteri and vaginae. Tritiated-thymidine LI studies supported this conclusion. It should be recognized that ^{3}H-thymidine incorporation did not differentiate DNA replication from DNA repair. However, all controls and experimental conditions reported in this study behaved normally with respect to known proliferative (DNA synthetic) response to estradiol. LIs of the stroma of EGFR-KO uterine and vaginal grafts treated with estradiol demonstrated a complete absence of proliferative response to estradiol. The growth deficiency of the EGFR-KO uterus and vagina could also be caused by a higher rate of apoptosis, although this was not measured in this study. Estradiol is known to inhibit apoptosis in granulosa cells of the rat ovarian follicle, and the lack of EGFR signaling here could inhibit estrogen-induced apoptosis. In any case, EGFR signaling seems to be required for optimal estrogen-dependent stromal growth in the uterus and vagina. Because the epithelium forms only about 10%, whereas the fibromuscular wall forms about 90% of the uterus (23), impaired growth of the stroma more profoundly affected overall organ size than impaired growth of the epithelium, which only constituted a small fraction of the uterus. Our results were consistent with a model in which the estrogen-receptor-mediated action of estradiol in either the epithelium or stroma elicited production of EGF ligands that subsequently interacted with the EGFR on stromal cells and stimulated stromal proliferation. The impaired stromal DNA synthesis in EGFR-KO mice indicated that estradiol by itself was not a complete mitogen for uterine or vaginal stromal cells, but instead that EGF ligands produced by the stromal cells and acting in an autocrine manner, or EGF ligands produced by the epithelium and acting as a paracrine manner, were involved in estrogen-induced growth of uterine and vaginal stromal cells.

Parallel studies in estrogen receptor-deficient (ERKO)/WT tissue recombinants clearly demonstrated that estradiol induction of uterine epithelial proliferation was a paracrine event requiring estrogen receptor-positive stroma (20). The current study extended these observations using EGFR-KO uteri and vaginae. We found that estrogen-induced epithelial growth mediated by stromal estrogen receptors was normal, suggesting that estrogen-induced stroma-mediated epithelial growth did not require the EGFR signaling pathway in the epithelial cells. Furthermore, the results of this study indicated that a functional stromal EGFR pathway was not required for stromal production of estrogen-induced paracrine factors necessary for epithelial cell growth but could be more crucial for uterine and vaginal stromal growth. It was reported that the preimplantation uterus differentially expressed full-length (EGFR-fl) and a truncated (EGFR-tr) forms of the EGFR (24). The EGFR-fl is a fully functional receptor, whereas the EGFR-tr is a secreted protein, which is not thought to have any direct cell signaling capabilities. *In situ* hybridization studies indicated that EGFR-fl transcripts were found only in the uterine stroma and myometrium, but not in the epithelium, whereas EGFR-tr message was detected in all major uterine cell-types (24). These findings were corroborated by immunohistochemical results showing that EGFR was detected only in the uterine stroma, deciduum, and myometrium, but not in the uterine luminal or glandular epithelium of the early pregnant mouse (25). Additionally, EGF ligands were shown to bind to a variety of other erb-B receptors (26). Using *in situ* hybridization, erb-B2 mRNA was detected primarily in uterine epithelial cells on days 1–4 of pregnancy in the mouse with the highest level found on day 1 (27). Further analysis showed that ovariectomized mice, treated with estradiol, up-regulated erb-B2 expression in the UtE by 3.5-fold using a combination of RT-PCR and *in situ* hybridization (27). Thus, it was possible that in EGFR-KO mice, EGF ligands could still be important for uterine/vaginal epithelial growth by signaling through these other receptors. Given the complexity of the EGF ligand family, it was not surprising that deletion of a single growth factor gene, such as TGFα, did not compromise the health or fertility of TGFα-KO mice (28). Taken together, these data imply that UtE was not the direct target for the effects of EGF-type growth factors and that their mitogenic effects were actually mediated by paracrine mechanisms involving other uterine cell-types expressing EGFR. Arguing against this interpretation are *in vitro* studies that showed a direct effect of EGF upon isolated UtE (29, 30). Using a collagen gel culture system, dissociated uterine and vaginal epithelial cells responded to EGF with growth in a serum-free, defined culture medium (29, 30). Unlike the *in vivo* situation, however, estradiol did not stimulate growth for vaginal or uterine epithelial cells in a similar collagen gel culture system (31), so comparisons between *in vitro* and *in vivo* results were difficult to reconcile. In any case, we could not rule out the possibility that, in WT mice, EGFR signaling in the epithelium occurred and was involved in estrogen-induced uterine and vaginal epithelial proliferation *in vivo*. However, in EGFR-KO mice, other ligand-receptor systems could clearly compensate for the lack of EGFR in the epithelium. One such possibility was erb-B2, which is a receptor subtype, capable

of binding EGF-related ligands in uterine epithelial cell proliferation (27).

Several EGF ligands are produced in the uterus and vagina. Uterine and vaginal epithelial cells were stimulated by estradiol to produce EGF and/or TGFα (5, 32). However, in the uterus, EGF seemed to be secreted apically into the uterine lumen (32) and, thus, could not be available for interaction with EGFR in either stromal or epithelial cells. Similarly, vaginal epithelium (stimulated *in vivo* by estradiol) expressed TGFα transcripts in suprabasal cell layers (33), which again raised the possibility that EGF ligands produced by vaginal epithelium could be unavailable for interaction with stromal or epithelial EGFR. TGFα expression also was found in the mouse uterus during the periimplantation period (day 1–4 of pregnancy) in a cell-type specific manner (34, 35). By *in situ* hybridization and immunoblot analysis, TGFα (34) and proTGFα (35), respectively, were localized in the luminal and glandular epithelia on days 1–4 of pregnancy, and many of the stromal cells expressed TGFα on days 3–4 of pregnancy. In the uterus of ovariectomized adult rats, the production of HB-EGF was stimulated in uterine stromal cells by progesterone (P) or P followed by estradiol. Such hormonal treatments repressed HB-EGF expression in the UtE, whereas estradiol alone increased HB-EGF expression in the epithelium. For ovariectomized adult mice, coinjection of P plus estradiol stimulated HB-EGF expression in uterine stromal cells, as detected by *in situ* hybridization, whereas estradiol alone increased expression of HB-EGF only in the epithelium (36, 37). Amphiregulin was induced by P in the uterine luminal epithelium of ovariectomized mice (38), whereas TGFα expression was stimulated by DES in the uterine epithelial cells, with only a modest increase in TGFα expression in uterine stromal cells (32). Thus, EGF ligands were produced by both epithelial and stromal cells, and therefore, proliferation of uterine and vaginal stromal cells could be elicited via either autocrine or paracrine mechanisms.

Thus, our results (using an EGFR null mutant mouse) showed that EGFR signaling was required for estrogen-induced proliferation of uterine and vaginal stromal cells but challenged the generally accepted notion that the EGFR receptor signaling system was crucial for estrogen-induced proliferation of uterine and vaginal epithelial cells.

If EGFR were not needed for estrogen-induced epithelial growth, then some other signaling pathway(s) in the uterus and vagina was used to elicit epithelial mitogenesis mediated via estrogen receptors in the stroma. Of the many possible compensatory growth factor pathways which could play key roles in estradiol-induced epithelial growth, keratinocyte growth factor (KGF), HGF, and insulin-like growth factor 1 (IGF-1) were worthy of consideration.

KGF fits many of the criteria considered essential for a mesenchymal mediator of epithelial development. Uterine tissue from cycling and ovariectomized monkeys, treated with combinations of estradiol and P, expressed KGF mRNA, which was increased in animals in the luteal phase or in animals treated with P (39). Thus, KGF was suggested to be a P-induced, stromally-derived, progestomedin. We found that KGF, injected directly into newborn female mice, stimulated uterine epithelial growth (unpublished results). However, KGF was highly induced after incisional wounding of the skin (40–42) and the bladder (43). Thus, it was perhaps worth considering whether the apparent induction of KGF in the uterus by P was secondary to apoptotic damage associated with reduced estrogen levels in the luteal phase. In any case, KGF could be an important paracrine mediator for uterine epithelial growth.

Hepatocyte growth factor (HGF, scatter factor) is mitogenic for epithelial cells of a number of estrogen-sensitive organs, including the mammary gland (44–46) and uterus (45). HGF transcripts were detected by RT-PCR in the adult mouse uterus, and c-met mRNA expressed in the UtE by *in situ* hybridization (45). HGF expressed in mammary stromal cells, stimulated ductal branching, and inhibited production of secretory proteins in organ culture (45). Proliferation of primary mouse mammary epithelial cells was stimulated by coculture with primary mouse mammary fibroblasts that produced HGF (44). Thus, HGF is another potential mediator of stromal effects on epithelial growth in the female genital tract.

IGF-1 also has been suggested to be a mediator of estrogen-stimulated proliferation in the uterus. IGF-1 and IGF-1 receptor expression were up-regulated in response to estrogen (47–49). Immature rats, implanted sc with pellets containing estradiol, exhibited an elevation in uterine IGF-1 and IGF-1 receptor mRNA after 72 h of treatment (50). Transgenic mice (homozygous for a null mutation of the IGF-1 gene) had thin, flaccid uteri with a wet weight only 13% that of WT mice (51). Another transgenic mouse, overexpressing IGF-binding protein-1 showed a significant reduction in both estradiol- and IGF-1-induced uterine DNA synthesis, compared with WT mice (52). IGF-1 expression was found in the uterine glandular and luminal epithelial cells on days 1–2 of pregnancy, whereas stromal cells, on days 3–4 of pregnancy and decidual cells on days 5–6 of pregnancy seemed to be the predominant sites of IGF-1 production (53). Treatment of ovariectomized mice with P and/or estradiol induced IGF-1 expression. Estradiol specifically induced IGF-1 in uterine epithelial cells, whereas P induced IGF-1 in stromal cells (53). The combination of both estradiol and P further stimulated IGF-1 expression in the uterine stroma. Taken together, these studies suggested that KGF, HGF, and IGF-1 were important for estradiol-induced epithelial growth and that they could compensate for KO of the EGFR signaling pathway.

We conclude that EGFR signaling was required for estrogen-induced uterine and vaginal stromal growth but not for estrogen-induced growth of uterine or vaginal epithelium. The resulting impaired stromal growth in EGFR-KO mice led to overall organ hypoplasia. Given the more extensive contribution of mesenchyme to the overall mass of the uterus, and the fact that uterine and vaginal mesenchyme played a central role as an inducer of uterine and vaginal development (54), the absence of EGFR signaling in the mesenchyme of EGFR-KO mice seemed to account, in large part, for the generalized hypoplasia of estrogen-sensitive female genital tract organs. Although EGFR signaling could be used during estrogen-induced uterine and vaginal epithelial proliferation *in vivo* in wild-

type mice, other, more important growth factor systems could act in conjunction with EGF to elicit epithelial growth. Clearly, other ligand-receptor systems did compensate for the lack of EGFR function in estrogen-induced proliferation of uterine and vaginal epithelia.

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Activation and Function of the Epidermal Growth Factor Receptor and erbB-2 during Mammary Gland Morphogenesis[1]

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Abstract

The hormonal stimulation of mammary gland morphogenesis is believed to occur through growth factor receptor signaling pathways. To determine the importance of the epidermal growth factor receptor (EGFR) pathway, we examined extracts of inguinal mammary glands from prepubertal and pubertal mice for tyrosine-phosphorylated EGFR and other erbB receptors. Tyrosine phosphorylation of both EGFR and erbB-2 was detected in normal female BALB/c mice at 5–6 weeks of age, but not during the prepubertal stage, *e.g.*, 24 days of age. Treatment of mice with estradiol or epidermal growth factor also stimulated the formation of mammary EGFR/erbB-2 phosphotyrosine. Waved-2 mice, which have impaired EGFR kinase activity, exhibited less mammary development than did wild-type (wt) mice when both were evaluated at 36 days of age. Because EGFR knockout (KO) mice die shortly after birth, glands from the newborns were implanted under the renal capsules of female nude mice. Under these conditions, extensive ductal growth was observed in mammary glands from wt animals; in contrast, glands from EGFR KO mice failed to grow beyond rudimentary structures. Tissue recombinants revealed that the wt fat pad supported the morphogenesis of EGFR KO epithelium, whereas the EGFR KO fat pad did not. Taken together, these data suggest that EGFR is essential for morphogenesis of the mammary ducts and functions during this period of mammary development as a heterodimer with erbB-2 in the mammary stroma.

Introduction

The hormonal activation of local growth factor pathways is considered a major stimulatory event for morphogenesis of the mammary gland (1). Previous studies have shown that estradiol is essential for ductal morphogenesis, and that this ovarian steroid functions by acting directly on the gland (2–4). Normally, ductal growth of the female mouse mammary gland is initiated around 4 weeks of age by the formation of terminal end buds and the ramification and elongation of the ductal tree (2). During the period of gland morphogenesis, marked DNA synthesis is observed in the outer (cap) cell layer of the end bud (5). The depletion of ovarian steroids by ovariectomy causes regression of the end buds and a cessation of ductal growth. When pellets containing nanogram amounts of estradiol are placed in the mammary FP[4] in proximity to the regressed ducts, end buds reappear in the treated glands, but not in the contralateral glands (3). Conversely, when pellets containing the estrogen receptor antagonist ICI 164,384 are placed in the gland, ductal formation is inhibited (4). The critical role of estradiol in the ductal growth of the mammary gland has been confirmed in female mice with a disrupted estrogen receptor gene in which ductal growth is severely impaired (6). Recent tissue recombinant studies in the uterus (7) and mammary gland (8) demonstrate that the epithelial estrogen receptor is neither necessary nor sufficient for estrogen-induced uterine epithelial proliferation or mammary ductal growth and branching. Instead, both of these events are paracrine processes dependent on estrogen receptor in the stroma.

The capacity for slow-release implants of the EGFR ligands EGF or TGF-α to stimulate the formation of end buds in the regressed glands of ovariectomized mice suggests that the EGFR is linked to a mitogenic pathway in the mammary gland (5, 9). When insulin, platelet-derived growth factor, or transferrin is substituted for EGF, a growth stimulus is not observed (10). Specific binding of ^{125}I-labeled EGF or EGFR immunoreactivity can be readily detected in the mammary gland of intact and ovariectomized mice (5, 11–13), which suggests that activation of the EGFR in this organ is probably not determined by hormonal regulation of EGFR levels. TGF-α and amphiregulin, another EGFR ligand, have been detected in the normal mouse mammary gland (9, 14,

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[4] The abbreviations used are: FP, fat pad; EGF, epidermal growth factor; EGFR, EGF receptor; KO knockout; TGF-α, transforming growth factor α; P-Tyr, phosphotyrosine; wt, wild-type.

15). Several studies with breast cancer cell lines indicate that estradiol can stimulate the level of mRNA or protein for some EGFR ligands. For example, production of TGF-α immunoreactivity and amphiregulin mRNA is increased in mammary cells treated with estradiol (16–18). When nude mice containing xenografted tumors derived from MCF-7 cells are no longer exposed to estradiol, a marked decrease is observed in tumor TGF-α mRNA but not in actin mRNA (19). Moreover, estrogens, but not other classes of steroids, stimulate the expression of a reporter gene linked to a 1.1-kb 5'-flanking region of the human TGF-α promoter that is transfected into estrogen receptor-positive breast cancer cell lines (20).

Cumulatively, these studies support the hypothesis that EGFR participates in mammary ductal growth. Thus, a major aim of the present study is to determine, within the context of the intact gland, whether EGFR is required for hormone-stimulated ductal growth and branching morphogenesis. Initially, we examined mammary gland extracts for changes in EGFR tyrosine phosphorylation that might parallel development or occur as a result of hormone exposure. Because EGFR can form a noncovalent functional homodimer as well as a heterodimer with other members of the erbB family, depending on the nature of the cell and ligand (21), we attempted to identify a potential erbB complex in the mammary gland that is activated as a consequence of hormone action. To further ascertain a functional role for mammary EGFR, we also examined the morphogenesis of glands obtained from waved-2 and EGFR KO mice. Targeted disruption of the mouse *Egfr* gene was previously shown to produce abnormalities in multiple organs, depending on the genetic background (22–24). The pronounced epithelial hypoplasia observed in many organs of EGFR KO mice was not observed in TGF-α KO mice (25) or in waved-2 mice that have a point mutation (Val-743-Gly) in the EGFR kinase domain resulting in reduced kinase activity (26, 27). The phenotype for EGFR KO mice suggests that this receptor has an important role in epithelial cell proliferation for many organs. Using tissue recombination techniques with mutant and wt mammary epithelium and stroma, we sought to determine whether ductal morphogenesis is dependent on EGFR signaling in the epithelium, stroma, or both. Because normal epithelial-stromal (paracrine) interactions play a critical role in hormone-induced responses in target organs (28, 29), we provide an appropriate context to evaluate the functions of receptor kinase(s) by using intact or reconstructed glands in these experiments.

Results

EGFR and erbB-2 Are Tyrosine Phosphorylated during Mammary Gland Morphogenesis. First we sought direct evidence for the activation of members of the erbB family of receptor tyrosine kinases *in vivo* during development or as a result of hormone exposure by measuring a specific increase in receptor tyrosine phosphorylation. We used extraction conditions similar to those previously reported to evaluate EGFR tyrosine phosphorylation *in vivo* (30). Immunoprecipitates of erbB proteins from female BALB/c mouse mammary glands were analyzed for P-Tyr before and during the period of mammary gland morphogenesis. As shown in Fig. 1, mammary glands of prepubertal 24-day-old mice contained both EGFR and erbB-2 but showed little, if any, tyrosine phosphorylation at this quiescent stage of mammary development. However, 12 of 37 animals analyzed between days 37 and 42, during the period of ductal morphogenesis, exhibited strong P-Tyr immunoreactivity associated with mammary EGFR and erbB-2. Moreover, all samples that showed intense EGFR P-Tyr bands also showed strong erbB-2 P-Tyr bands (Fig. 1, *A* and *B*). The variation in EGFR/erbB-2 P-Tyr content among the different animals during the period of morphogenesis probably reflects the influence of a cyclical secretion of ovarian estrogens. All animals analyzed between days 37 and 42 were in an estrous cycle, whereas those at 23 or 24 days of age were not. Morphology, cell composition, and the incorporation of [^{3}H]thymidine in rat mammary terminal end buds were previously shown to vary with the different phases of the estrous cycle (31). Immunoreactivity for erbB-3 and erbB-4 was detected in the mammary glands of prepubertal and subadult female mice; however, these receptors did not reveal a specific increase in P-Tyr during the period of ductal morphogenesis (Fig. 1, *C* and *D*).

Estradiol Stimulates Tyrosine Phosphorylation of Mammary EGFR and erbB-2. Ovariectomy of mice during the period of morphogenesis inhibits gland development, which can be restored by treatment with estradiol (2). Mice ovariectomized at 30 days of age were treated with estradiol for 7 days beginning at 45 days of age. When we examined immunoprecipitates of erbB receptors from the mammary glands of vehicle- and hormone-treated castrates for changes in P-Tyr content, we found that the level of each erbB protein was comparable between control (vehicle-treated) and hormone-treated groups. However, there was a marked increase in P-Tyr content in immunoprecipitates of EGFR and erbB-2 from the glands of animals treated with estradiol when compared with that in immunoprecipitates from controls (Fig. 2). Treatment with estradiol did not increase the tyrosine phosphorylation of erbB-3 (data not shown).

EGF Rapidly Stimulates Tyrosine Phosphorylation of EGFR and erbB-2 in Mammary Stroma. The capacity for EGFR ligands to stimulate tyrosine phosphorylation of both EGFR and erbB-2 in cells expressing these proteins has been attributed to ligand-induced heterodimerization and transphosphorylation by these receptors (32–35). To assess whether the formation of EGFR/erbB-2 complexes contributes to the coordinated phosphorylation of both receptors in response to estradiol, we treated mice ovariectomized at day 30 with EGF and evaluated the immunoprecipitates of these receptors from mammary extracts for P-Tyr. Fifteen min after treatment with EGF, a marked increase in P-Tyr was observed in both receptors (Fig. 3, *A* and *B*). Under our experimental conditions, immunoprecipitates containing the ligand-induced EGFR P-Tyr did not coimmunoprecipitate erbB-2 (data not shown). To determine whether the EGFR/erbB-2 P-Tyr observed after treatment with EGF occurs in the FP devoid of ductal structures, inguinal mammary glands from mice castrated prepubertally were divided in half to obtain ductal epithelium (proximal) and duct-free (distal) FP specimens. Accordingly, tyrosine phosphorylated EGFR and

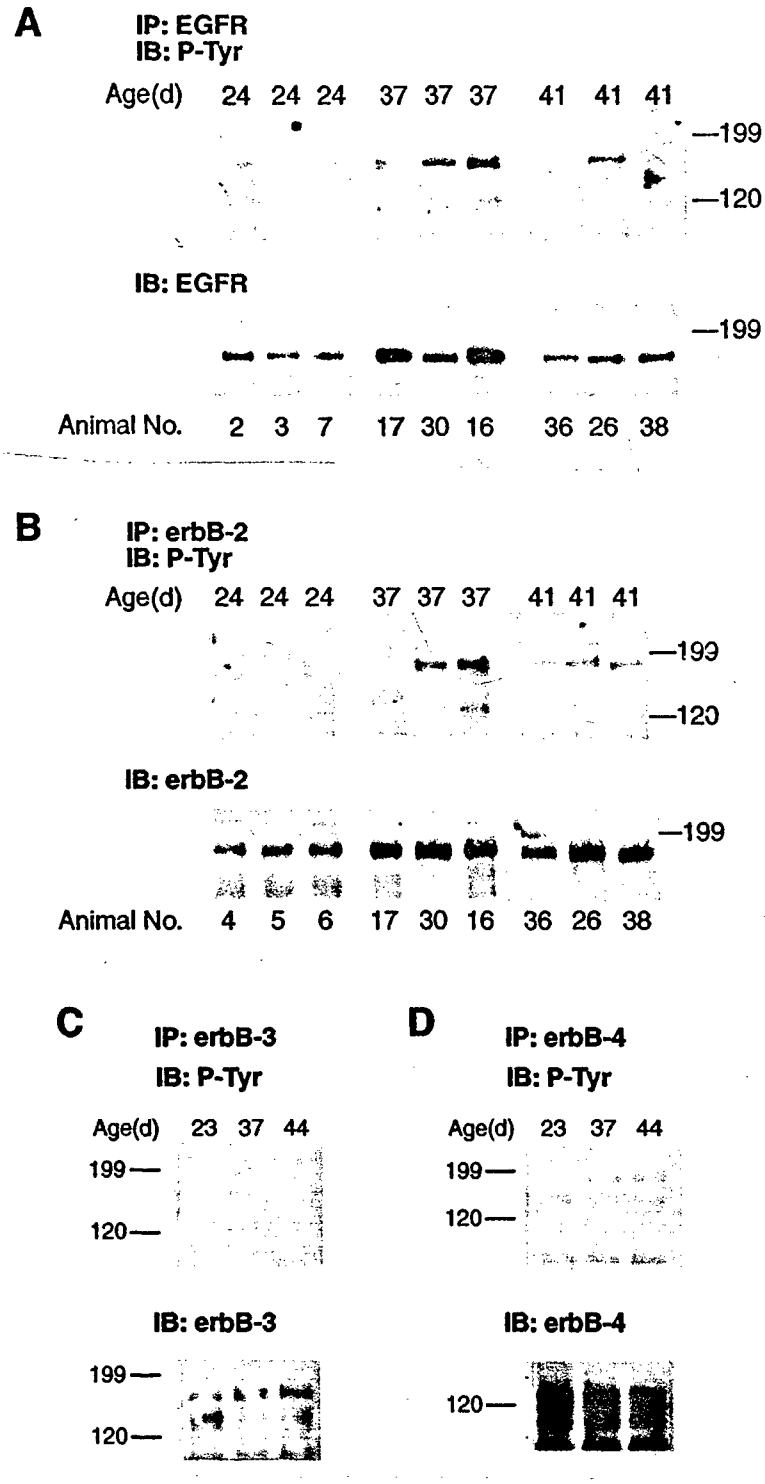

Fig. 1. Tyrosine phosphorylation of BALB/c mouse mammary EGFR and erbB-2 during ductal morphogenesis. Mammary glands from prepubertal mice at 23 or 24 days (*d*) of age or from cycling mice at 37, 41, or 44 days of age were extracted and analyzed for erbB proteins as described in "Materials and Methods." Aliquots of the extracts were incubated with antibody to the erbB protein, and immunoprecipitates (*IP*) were evaluated by immunoblotting (*IB*) with antibody to P-Tyr or the specific erbB. In *A* and *B*, only one erbB member for day 24 EGFR or erbB-2 was analyzed per mouse because of the low amount of mammary tissue. For days 37 and 41, both EGFR and erbB-2 were analyzed for each mouse. For 37- or 41-day-old mice, the extent of EGFR/erbB-2 P-Tyr was markedly different among most animals analyzed; however, for each animal analyzed, EGFR P-Tyr varied directly with that of erbB-2 P-Tyr. In *C* and *D*, glands from four (day 23) or three (day 37 or day 44) animals were pooled. The pooled glands from day 37 or day 44, but not those from day 23, also revealed positive EGFR/erbB-2 P-Tyr (data not shown).

erbB-2 were present in both the proximal and distal compartments after treatment with EGF, which indicates that an EGFR/erbB-2 complex can form in the stromal cells of the FP in response to an EGFR ligand (Fig. 4, *A* and *B*). Relevant to this finding, EGFR immunoprecipitates prepared from omentum fat in these animals also exhibited P-Tyr after an injection of EGF (data not shown).

Mammary Gland Morphogenesis Is Impaired in the Waved-2 Mouse. Examination of mouse mammary gland whole mounts provides a convenient method to determine whether a specific mutation can affect the normal pattern of ductal elongation and branching that occurs during the morphogenesis of this organ. To assess the effects of the waved-2 EGFR mutation on mammary morphogenesis,

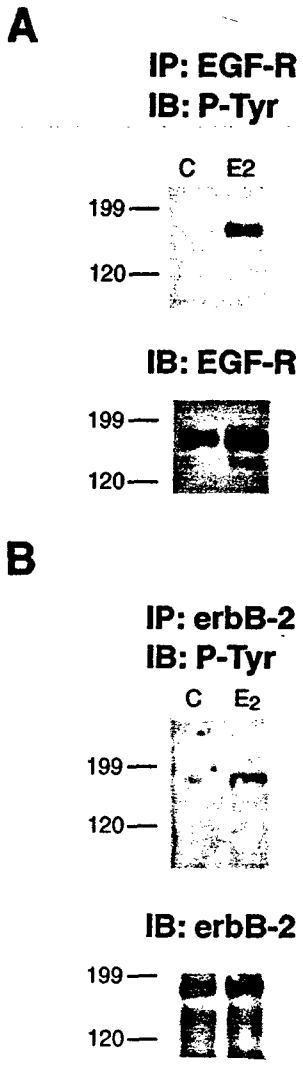

Fig. 2. Estradiol stimulates tyrosine phosphorylation of mouse mammary (*A*) EGFR and (*B*) erbB-2. BALB/c mice were ovariectomized at 30 days of age and treated s.c. 2 weeks after castration. Mammary extracts were prepared after treatment with 1 μg of estradiol (E_2) or vehicle (*C*) daily for 7 days. Immunoprecipitates (*IP*) were evaluated by immunoblotting (*IB*) with specific antibody to P-Tyr or the specific receptor.

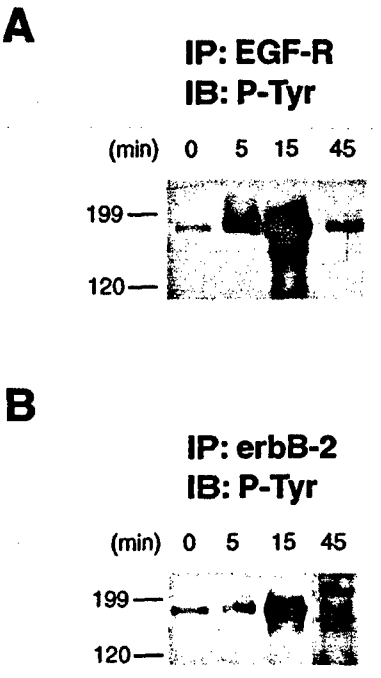

Fig. 3. EGF rapidly stimulates tyrosine phosphorylation of mammary (*A*) EGFR and (*B*) erbB-2. BALB/c mice ovariectomized at 30 days of age were injected s.c. with a single dose of EGF (75 μg) 2 weeks after castration. Immunoprecipitates (*IP*) were prepared from the mammary organs obtained at the indicated time periods after treatment with EGF. Immunoprecipitates were evaluated by immunoblotting (*IB*) with the specific antibody to P-Tyr or with the receptor protein.

whole mounts of the inguinal glands of these mice at 36 days of age were compared with those of age-matched females of the same genetic background. Fig. 5*A* reveals the typical ductal branching pattern of the subadult mouse mammary gland, in which some of the advancing ducts with terminal end buds have extended into the FP well past the lymph node. In contrast, whole mounts of the waved-2 gland, as depicted in Fig. 5*B*, revealed fewer branching structures with terminal end buds. Some of the waved-2 ducts appear to be dilated, and the terminal end buds appear to be enlarged when compared with those of the wt gland. By 100 days of age, the mammary ducts of both control and waved-2 mice had extended to the limits of the FP (data not shown).

Stromal EGFR Is Required for Mammary Ductal Morphogenesis. Mice with a targeted disruption of the EGFR provide an appropriate animal model to determine the importance of this erbB family member in mammary morphogenesis. Because homozygotes with this mutation die *in utero* or neonatally (22, 24), intact mammary organ rudiments were grafted beneath the renal capsule of adult female nude mice. Grafts of intact neonatal mammary rudiments (epithelium plus FP) from EGFR KO female mice displayed limited ductal growth and branching, leaving most of the area of the FP unoccupied (Fig. 6). However, such grafts of wt mammary glands developed an extensive network of epithelial ducts that filled the entire FP (Fig. 6).

To determine whether the impaired growth of EGFR KO mammary glands was due to an absence of EGFR in the epithelium and/or FP, mammary tissue recombinants were prepared with mammary ductal epithelium (E) and FP from wt and EGFR KO mice (wt-FP + wt-E, wt-FP + EGFR KO-E, EGFR KO-FP + wt-E, and EGFR KO-FP + EGFR KO-E) and grafted into intact female nude mice. After 1 month of *in vivo* growth, tissue recombinants constructed with wt-FP exhibited extensive ductal growth (Fig. 7, *A* and *C*), regardless of whether the epithelium originated from wt or EGFR KO mammary glands (wt-FP + wt-E or wt-FP + EGFR KO-E). Conversely, tissue recombinants constructed with EGFR KO-FP did not support ductal growth when either wt-E or EGFR KO-E was used (EGFR KO-FP + wt-E or EGFR KO-FP + EGFR KO-E; Fig. 7, *B* and *D*). In all tissue recombinants containing EGFR KO-FP, ductal growth was similar to that seen in intact EGFR KO mammary gland grafts; invariably, vast areas of the FP remained unoccupied.

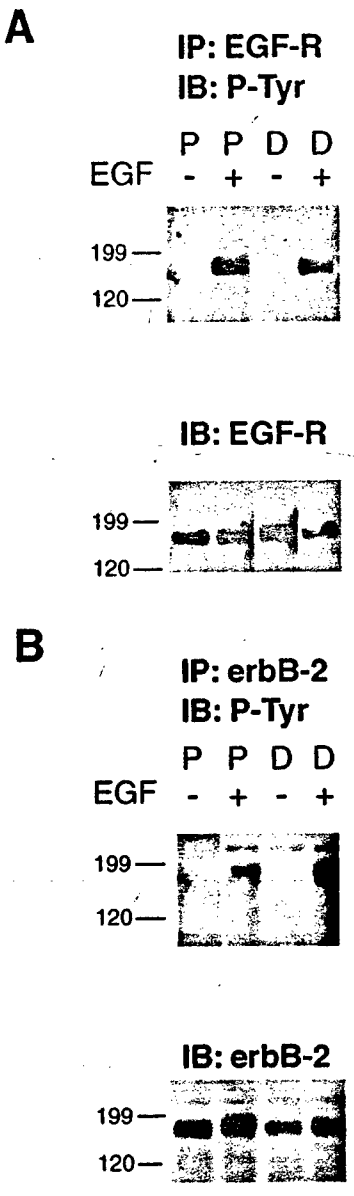

Fig. 4. EGF stimulates tyrosine phosphorylation of (*A*) EGFR and (*B*) erbB-2 in the proximal (*P*) and distal (*D*) aspects of the mammary organ. BALB/c mice ovariectomized at 20 days of age were injected with a single dose of EGF (75 μg) 2 weeks after castration. Ten min after treatment with the growth factor, the organ was divided in half to obtain duct-containing (*P*) and duct-free (*D*) aspects. Immunoprecipitates (*IP*) from mammary extracts were evaluated by immunoblotting (*IB*) with the antibody specific to P-Tyr or with the receptor protein.

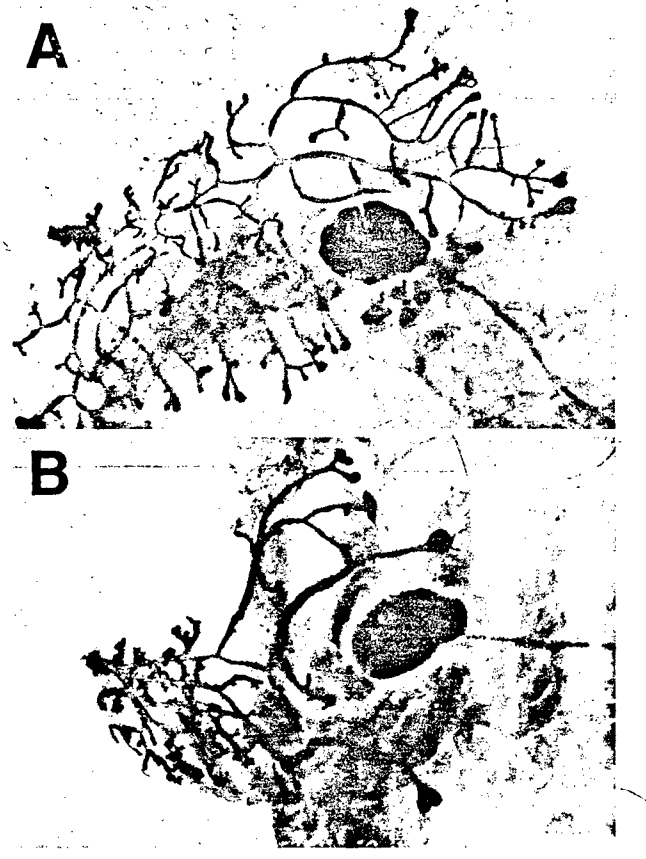

Fig. 5. Whole mounts of mammary glands from waved-2 and wt (control) mice at 36 days of age are shown. Whole mounts of the inguinal mammary glands were prepared and stained with toluidene blue. At this age, the wt mammary gland (*A*) exhibits the characteristic branching ductal tree that has advanced beyond the lymph node. In contrast, the glands from waved-2 mice (*B*) reveal less branching and extension into the FP. In addition, the terminal end buds and ducts of the mutant animals appear to be dilated or swollen.

Discussion

The appropriate experimental model to dissect the complex interplay between the estrogen receptor and growth factor pathways requires the normal complement of epithelial and stromal cells that constitute the hormonally responsive mammary gland. For this reason, the present study used *in vivo* models of intact mammary gland of normal and mutant mice, mammary gland grafts, and mammary tissue recombinants. In all three models, normal architecture and cell-cell interactions are maintained, thereby providing an appropriate milieu in which estrogens converge with growth factor-regulatory pathways in the mammary gland. Our observation that tyrosine phosphorylation of EGFR and erbB-2 increases as the mammary gland progresses from the prepubertal stage (day 24) to the cycling subadult stage further corroborates that the activation of these kinases is important for ductal growth. The cumulative data of the present study support but do not prove that the EGFR/erbB-2 heterodimer is the functional form by which these receptors transduce signals during mammary ductal morphogenesis. This concept could account for why the intensity of tyrosine phosphorylation of EGFR varied directly with that of erbB-2 during the period of morphogenesis. The heterodimeric potential of mammary EGFR/erbB-2 is also supported by the colocalization of these proteins in cells of the mouse mammary gland, as determined by immunocytochemistry (13). The fact that treatment of young castrates with estradiol or EGF stimulated tyrosine phosphorylation of both EGFR and erbB-2 suggests that a hormone-induced increase in

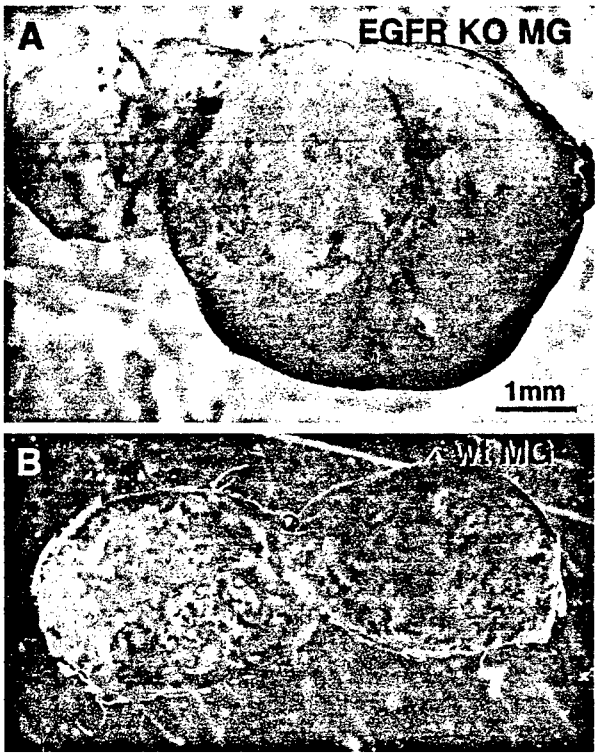

Fig. 6. Whole-mount preparations of grafted neonatal mammary glands (*MG*) from (*A*) EGFR KO and (*B*) wt mice are shown. Two inguinal mammary glands from EGFR KO or wt mice were grown for 1 month beneath the kidney capsule in intact female nude mice and have fused together. Grafted mammary rudiments originated from 0–3-day-old EGFR KO and wt mice.

local availability of an EGFR-specific ligand, such as TGF-α or amphiregulin, is required for tyrosine phosphorylation of the heterodimer. On this basis, it would be reasonable to expect that mutations that partially impair EGFR kinase activity, as seen in the waved-2 mouse, would result in a reduction of erbB-2 transphosphorylation and its interaction with downstream signaling components. Because the mammary gland of the waved-2 mouse was less developed than that of the age-matched wt mouse during the period of morphogenesis, it is likely that EGFR kinase activity is functionally important for the ductal growth program. Moreover, the profound impairment of ductal and branching morphogenesis in the transplanted EGFR KO mammary glands supports the essentiality of EGFR for mammary development. The neonatal lethality and significant phenotypic changes reported previously in EGFR KO mice (22, 24) demonstrate that functional redundancy does not occur among the erbB members *in vivo*, *i.e.*, the loss or impairment of one member is not supplanted or compensated for by another member.

Our tissue recombinant studies with EGFR KO and wt mice suggest that the specific mammary stromal cell populations that exhibit EGFR/erbB-2 activation are also essential for ductal morphogenesis. Earlier studies (5, 11, 12) have reported specific stromal binding sites for ^{125}I-labeled EGF on sections of the mouse mammary gland. Binding sites were apparent on both fibroblasts near the neck of the terminal end bud and adipocytes (5, 11); both of these sites stained positively with antisera to EGFR and erbB-2 (13). EGFR also resides in the proliferating epithelial cap cells of the end bud, as demonstrated by both the binding of ^{125}I-labeled EGF (5, 9) and immunocytochemistry (13), but our present tissue recombinant experiments suggest that the receptor in the epithelium is not essential for ductal elongation. Instead, we propose that hormonal activation of EGFR/erbB-2 in stromal cells near the terminal ends of the ducts is important for mammary ductal growth.

The downstream pathways and changes in gene expression that emanate from the activated EGFR/erbB-2 heterodimer are currently unknown, but it is conceivable that the synthesis of extracellular matrix components, growth factors, lipid mediators, and proteases could be influenced by this active complex. The permissive stromal-epithelial interactions that occur during mammary ductal growth, including those originating from the hormonal activation of stromal EGFR/erbB-2, can probably occur in other adipose tissues, such as the pararenal FP, because these ectopic sites are known to support mammary ductal growth (2, 36). On the basis of the known stromal occurrence of the estrogen receptor in the developing gland (3) and the requirement of mammary epithelium for the expression of some ligands (37), the pathway between hormone-hormone receptor interaction and ligand activation of EGFR/erbB-2 may occur as an interplay of epithelial and stromal signals.

The detection of immunoreactivity for erbB-3 and erbB-4 is in accord with an earlier study (37) that reported transcripts for these erbB members in the postnatal mouse mammary gland. However, our inability to detect tyrosine-phosphorylated forms of mammary erbB-3 and erbB-4 in the intact animal indicates that these receptors may not be essential for ductal morphogenesis. The engagement of one or both of these receptors may occur at another stage of mammary gland development, *e.g.*, lobuloalveolar growth. Indeed, when we examined the extracts of mammary glands from mice at late pregnancy (days 14–18), tyrosine-phosphorylated erbB-2 and erbB-3 were detected.[5] This agrees with the enhanced expression of heregulin transcripts at this stage of pregnancy (37). The EGF-like heregulin binds to erbB-3 and erbB-4, but not to EGFR or erbB-2, and it can stimulate heterodimers such as that of erbB-2/erbB-3 (21, 38–40). Thus, a stage-specific activation of different erbB members, probably functioning as heterodimers, occurs during the course of mammary gland development and differentiation, with ligand expression being an important variable in determining what receptor combinations are recruited for signaling.

In summary, our findings enable us to postulate that the formation of an active EGFR/erbB-2 heterodimer in mammary stromal cells occurs as a response to estrogen action and is an early signaling event for ductal morphogenesis.

[5] P. P. DiAugustine, M. P. Walker, N. Kenney, and R. G. Richards, unpublished data.

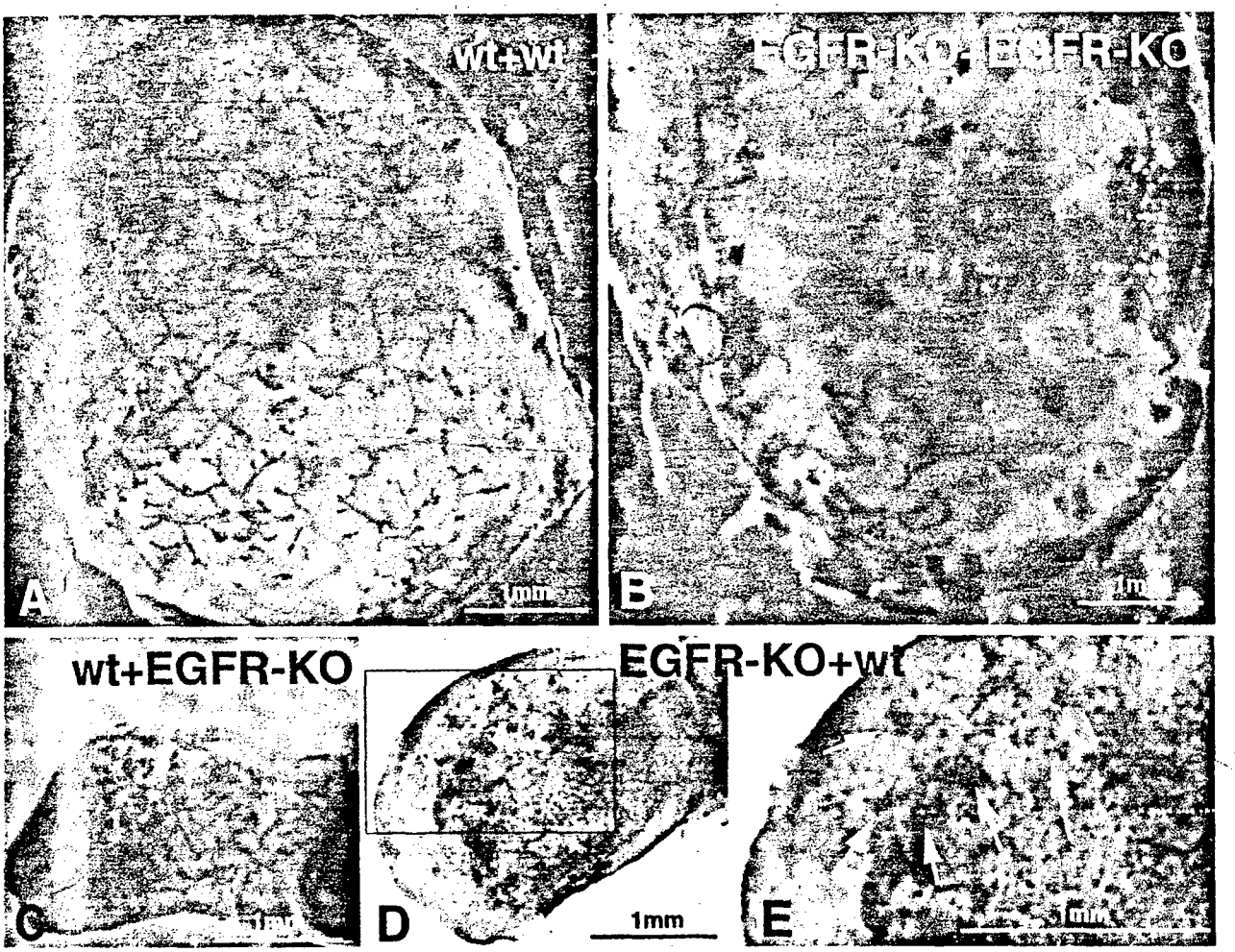

Fig. 7. Whole-mount preparations of individual mammary tissue recombinants constructed with FPs and epithelial ducts (*E*) of wt and EGFR KO mammary rudiments of 0–3 day-old mice are shown. All tissue recombinants were grown for 1 month under the renal capsule of intact female nude mice. *A*, wt-FP + wt-E recombinant; *B*, EGFR KO-FP + EGFR KO-E recombinant; *C*, wt-FP + EGFR KO-E recombinant; *D*, EGFR KO-FP + wt-E recombinant. The enclosed area in *D* was magnified and shown in *E*. The *arrows* highlight the rudimentary gland that was observed with this tissue recombinant. In those cases (*B* and *D*) in which EGFR KO-FP was used to construct the tissue recombinant, the mammary ducts failed to extend and branch into the FP and remained, instead, as rudimentary structures.

Further application and refinements of the approaches used in this study both in the intact animal and *in vitro* may ultimately lead to the identification of downstream regulatory pathways that contribute to normal mammary growth and mammary tumor development or progression.

Before our experiments were completed, a separate laboratory reported on transgenic mice with a dominant negative COOH-terminal-truncated EGFR under the control of the mouse mammary tumor virus-long terminal repeat (41). The mammary glands in 5-week-old transgenic mice had much fewer and smaller terminal end buds than were observed in the glands of control littermates. Expression of the transgene during this period of development is uncertain, because neither the transgene product nor the wt EGFR protein could be detected in the mammary glands of virgin animals. By 17 weeks of age, the transgenic mice exhibited extensive mammary development but lacked the side branching observed in the wt mice. This study is confounded by the fact that the distribution, *e.g.*, epithelium *versus* stroma, of the transgene product is not likely to mimic that of the wt receptor.

Materials and Methods

Materials. Estradiol (17β-estradiol) was obtained from Sigma (St. Louis, MO). EGF (culture grade) was purchased from Collaborative Biomedical Products (Bedford, MA). Protein A-Sepharose was obtained from Pharmacia Biotech, Inc. (Uppsala, Sweden). The Xcell mini-cell for SDS-PAGE and the blot module for Western transfer were from Novex (San Diego, CA). Prestained molecular markers were from Bio-Rad (Melville, NY), and the polyvinylidene fluoride membrane was from Millipore (Immobilon-P; Bedford, MA). Enhanced chemiluminescence Western blotting reagents were obtained from Amersham (Arlington Heights, IL).

Antibodies. Horseradish peroxidase-conjugated anti-P-Tyr monoclonal antibody was obtained from ICN Biomedicals, Inc. (PY20; Irvine, CA). A rabbit polyclonal antiserum was raised against a peptide corresponding to the extreme COOH-terminal region of the rat EGFR (residues 1157–1186) and was affinity-purified (42). Rabbit polyclonal antibodies directed against synthetic peptides corresponding to the COOH-terminal

region of erbB-2 (residues 1169–1186), erbB-3 (residues 1307–1323), and erbB-4 (residues 1291–1308) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Treatments and Preparation of Tissue Extracts. BALB/c mice (Charles River Laboratories, Raleigh, NC) were ovariectomized at 20 or 30 days of age and treated s.c. 2 weeks after castration. Castrated mice received seven daily injections of 1 μg of estradiol in sesame oil or a single injection of 75 μg of EGF [0.1 ml PBS-1% (w/v) bovine albumin], and mammary tissues were removed at various times after treatment; control mice received the vehicle only. For developmental time course experiments, the fourth inguinal mammary glands were collected from prepubertal or cycling subadult BALB/c mice. To encourage cycling, cage bedding from male mice was added to that of females when the latter reached 30 days of age. Vaginal smears were performed periodically to confirm the occurrence of an estrous cycle. Tissue extracts were prepared as described previously (30, 42) with the following modifications: mammary glands (15% wet weight/volume) were disrupted at 4°C in buffer A [1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 50 mM Na_2MoO_4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 15 μM 4-amido-phenylmethylsulfonyl fluoride in 20 mM HEPES (pH 7.4)] with three 10-s bursts of a Brinkman Polytron at the highest setting. After centrifugation at 21,000 × *g* for 4 min, the extract was analyzed as described below. Protein concentration was determined by the Pierce BCA method.

Immunoprecipitation and Western Blot Analysis. Aliquots of mammary extracts (300–400 μg of protein) were added to buffer B [150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM Na_3VO_4, 20 mM NaF, 50 mM Na_2MoO_4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 15 μM 4-amido-phenylmethylsulfonyl fluoride in 50 mM Tris (pH 8.5)] and incubated with specific antibody followed by the addition of protein A-Sepharose. Immunoprecipitates were washed three times with buffer B, boiled in Laemmli buffer for 5 min, and stored at −20°C. After thawing, the proteins were boiled for an additional 2 min, resolved by SDS-8% PAGE, and transferred to Immobilon-P membrane. The membrane was blocked with Tris-buffered saline, 0.1% Tween 20, and 5% bovine albumin and probed with the appropriate primary and secondary antibodies. Immunoreactive proteins were detected using enhanced chemiluminescence.

Whole Mounts and Mammary Organ Transplants. Young female homozygous waved-2 (*wa-2*) mice (B6EiC3H-a/A-Egfr^{wa2}Wnt3a^{vt}) and nonmutant female controls (B6EiC3H-a/AF1) were purchased from The Jackson Laboratory (Bar Harbor, ME). Whole mounts of the mammary glands from age-matched animals were prepared as described previously (43).

At birth, the mammary duct of the female mouse has six to eight terminal ducts that are just beginning to invade the edge of the FP. To isolate the neonatal mammary gland, neonates were sacrificed by decapitation, and the skin was peeled back to expose the number 4 mammary gland. The main duct of the gland was severed at the nipple, and the entire mammary gland (ducts plus the FP) was dissected from the skin (8). The methods for the development and phenotypic identification of EGFR KO mice with C57/6JX Swiss Webster black background were described previously (24). Because most EGFR KO mice die shortly after birth, the investigation of ductal growth in wt *versus* EGFR KO mammary glands was accomplished by grafting complete mammary rudiments (epithelium plus FP) beneath the renal capsule of female athymic nude mice. Mammary FPs and cleaned main epithelial ducts of wt and EGFR KO mice were used to prepare the four tissue recombinants (wt-FP + wt-E, wt-FP + EGFR KO-E, EGFR KO-FP + EGFR KO-E, and EGFR KO-FP + wt-E). To prepare the tissue recombinants, isolated mammary rudiments were transilluminated under a dissecting microscope, so that the FP could be bisected just in advance of the invading epithelial ducts to yield a FP free of epithelial tissue. The main duct was isolated by microdissection, trimmed to length (100–200 μm), and cleaned of extraneous stromal tissue with microforceps. Mammary FPs and cleaned main epithelial ducts of wt and EGFR KO mice were transferred to nutrient agar plates (44), and a segment of the main duct was placed into a pocket created in the FP using microforceps. After an overnight culture to allow the tissues to adhere, the tissue recombinants were grafted under the renal capsules of athymic female nude mice [see the mammary gland Web site (http://mammary.nih.gov/) for details] and allowed to grow for 1 month.

Acknowledgments

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PARACRINE MECHANISMS OF MOUSE MAMMARY DUCTAL GROWTH

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1. INTRODUCTION

Ductal growth during puberty is stimulated by estrogens, which elicit their effects via specific estrogen receptors, ERα and ERβ. Analysis of mice with targeted disruption of ERα or ERβ has emphasized the importance of ERα in mammary gland development. In the mouse mammary gland, ERα are expressed in both epithelial and stromal cells (Kurita and Cunha, unpublished), which raises the possibility that the growth and morphogenetic effects of estrogen could be mediated via either epithelial or stromal ER. The aim of this paper is to review the role of epithelial versus stromal ER in mammary ductal-alveolar growth to assess the importance of paracrine mechanisms.

For many years it has been tacitly assumed that actions of estradiol and on epithelium are mediated directly through ER in epithelial cells themselves. Mammary ductal growth gland is induced by estradiol. The presence of ERα in mammary epithelium combined with the ability of estradiol to induce proliferation in mammary epithelium certainly suggests a causal link; i.e., that effects of estradiol on mammary epithelial proliferation are mediated directly through ER in these cells. However, such a causal link between the

presence of mammary epithelial ER and the effects of estradiol on proliferation of mammary epithelium has never been established. In fact, a rapidly emerging body of evidence suggests that this is clearly not true and that stromal ER mediates many effects of estradiol on estrogen target epithelium.[1]

Methodologies for producing αERKO mice, in which the ERα gene has been rendered non-functional by gene targeting,[2] has allowed examination of phenotypic and functional consequences of an absence of ERα.[3] We have recently developed a new experimental system, which utilizes tissues from ERKO mice to study the mechanism of estradiol action.[1] This system involves separating and recombining epithelial and stromal tissue from αERKO mice with that of a wild-type mice, which express ERα. This tissue separation/recombination technique provides a unique method for experimentally controlling the ERα status of both stroma and epithelium. Thus, tissue recombinations can be prepared, which lack ERα in both stromal and epithelial compartments, express ERα in either epithelium or stroma, or express ERα in both epithelium and stroma. Tissue recombinants are then transplanted into host animals. By analyzing effects of a lack of stromal and/or epithelial ERα on estradiol response such as ductal growth, the role of ERα in each tissue compartment can be definitively determined.

2. STROMAL ERα IS REQUIRED FOR DUCTAL GROWTH IN THE MOUSE MAMMARY GLAND

Ductal mammary growth is profoundly impaired in αERKO mice. By the end of puberty, the mammary fat pad (FP) was almost entirely filled with ducts in wild-type mice, while in αERKO mice most of the FP was devoid of ducts.[4] Analysis of tissue recombinants were prepared with mammary epithelium (MGE) and mammary fat pad (FP) of wild-type (wt) and αERKO mice demonstrated that tissue recombinants composed of wt-FP+wt-MGE developed an extensively branched ductal network, which completely filled the fat pad. In contrast, ductal growth was minimal in αERKO-FP+αERKO-MGE tissue recombinants. When αERKO mammary epithelium was grown in association with wild-type FP (wt-FP+αERKO-MGE), αERKO mammary epithelium underwent extensive ductal growth. Although there was some variability in the amount of ductal growth in individual tissue recombinants, ductal growth was always more extensive in wt-FP+wt-MGE and wt-FP+αERKO-MGE versus αERKO-FP+αERKO-MGE tissue recombinants. Surprisingly, ductal tissue was never recognized in wholemounts of αERKO-

FP+wt-MGE tissue recombinants. However, serial sections of αERKO-FP+wt-MGE tissue recombinants revealed that all contained small foci of mammary ducts.[4] These findings demonstrate that stromal fat pad cells are critical estrogen targets, and that estrogen elicits mammary ductal growth through stromal ERα. Epithelial ERα is neither necessary nor sufficient for ductal growth. These findings in mammary gland are in complete agreement with comparable tissue recombinant studies in the uterus and vagina.[1]

3. STROMAL EPIDERMAL GROWTH FACTOR RECEPTOR PLAYS A CRITICAL ROLE IN MAMMARY EPITHELIAL GROWTH

Growth factors such as epidermal growth factor are known to play a role in stromal-epithelial interactions which are critical in determining patterns of mammary growth, development, and ductal morphogenesis. To determine the role of signaling through the epidermal growth factor receptor (EGFR) in mammary ductal growth and branching, mice with a targeted null mutation in the EGFR were used. Such EGFR-KO mice die perinatally, and thus transplantation methods were used to study growth and development of EGFR-KO mammary glands.[5] When transplanted under renal capsules of athymic female mice, neonatal mammary glands of wild-type mice underwent extensive ductal growth with complete filling of the FP. Conversely, neonatal mammary glands of EGFR-KO mice exhibited impaired ductal growth with incomplete filling of the FP. These findings demonstrated that EGFR is essential for mammary ductal growth. To determine whether impaired ductal growth was due to an absence of EGFR in epithelium (E), FP or both, EGFR-KO or wild-type MGE was transplanted into wild-type gland-free FPs. Surprisingly, transplants of either EGFR-KO or wild-type MGE into wild-type mammary FPs exhibited extensive ductal growth with comparable complete filling of the FP in transplants into intact virgin female hosts. Similarly, lobulo-alveolar development was equivalent in transplants of EGFR-KO or wild-type MGE into wild-type mammary FPs, when the hosts also received a pituitary isograft as a source of prolactin.[5] These findings suggested that the absence of EGFR-signaling in the epithelium is not required for mammary ductal growth and lobulo-alveolar development. Instead, these findings suggested that the impaired ductal growth exhibited by EGFR-KO mammary glands was due to an absence of EGFR signaling in the mammary FP.

To determine the irrespective roles of stromal versus epithelial EGFR in mammary ductal growth, neonatal EGFR-KO and wild type (wt) mammary

glands were surgically separated into FP and main epithelial duct (E) and then recombined as follows: wt-FP + wt-E, wt-FP + EGFR-KO-E, EGFR-KO-FP + wt-E, and EGFR-KO-FP + EGFR-KO-E +. When tissue recombinants contained wild type stroma, ductal development proceeded regardless of the epithelial source (wt or EGFR). This outcome corroborates results of transplantation of epithelium into cleared FPs. However, when tissue recombinants contained EGFR-KO stroma, ductal growth was meager regardless of the epithelial source. These data indicate that signaling through EGFR must occur in the stroma surrounding the epithelial ducts to induce normal ductal proliferation and morphogenesis in the mammary gland.[5]

4. CONCLUSION

Mammary ductal growth is estrogen-dependent and is profoundly impaired in αERKO mice.[6] Tissue recombinant studies using FPs and mammary epithelia from αERKO and wt mice demonstrate that estrogen stimulates mammary ductal growth via a paracrine mechanism, acting through stromal ERα. Epithelial ERα is neither necessary nor sufficient for ductal growth.[4] EGFR signaling is thought to be a downstream effector of estrogen action in several estrogen target organs.[7] Mammary epithelium expresses EGFR, and epidermal growth factor and TGFα are mitogens for mammary epithelial cells.[8] Despite this, our data clearly show that ductal growth does not require signaling through epithelial EGFR in the mammary gland in vivo. Instead, EGFR is absolutely necessary in the stromal FP to induce estrogen-dependent ductal growth. Our results suggest that under stimulatory estrogenic conditions the stroma responds to estrogen action through an EGFR-mediated signaling event that is required for stimulation of epithelial growth and development. In contrast, epithelial EGFR and ERα are neither necessary nor sufficient for ductal growth. What are the gene targets of estrogen action in mammary epithelial cells? One target of estrogen action in mammary epithelium is upregulation of the progesterone receptor (PR), which is only detectable in mammary epithelium.[9] The importance of epithelial PR in the mammary gland is demonstrated as impaired lobulo-alveolar development in PR null mice.[10] The targets of EGFR signaling remain to be determined.

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Proteinases, Cell Cycle Regulation, and Apoptosis During Mammary Gland Involution (Minireview)

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Key Words: mammary gland; involution; proteinases; cell cycle; development

INTRODUCTION

An intact basement membrane is essential for the proper function, differentiation, and morphology of many epithelial cells. The remodeling of extracellular matrix (ECM) accompanies cell migration, morphogenesis, development, and tissue maintenance, as well as in pathological events including inflammation, fibrosis and tumor invasion. The balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) has been implicated in these processes. When active MMPs are higher than TIMPs, net degradation and erosion of ECM take place, whereas when TIMPs are higher than MMPs, net ECM accumulation ensues.

ECM not only provides structural support for cells within a tissue, but also plays a more active role by affecting adhesion, migration, proliferation, and differentiation of many cell types. Events that influence the dynamic state of the ECM must therefore play a critical role in both normal and pathological processes. One such event is the remodeling and degradation of the ECM by ECM-degrading proteinases. Normal physiological processes, such as involution of the mammary gland, require ECM remodeling (Basbaum and Werb, 1996; Coussens and Werb, 1996; Werb, 1997; Lochter et al., 1998). Likewise, pathological processes have been associated with excessive ECM degradation. These conditions include mammary fibrosis, tumor invasion, and metastasis (Ashkenas et al., 1994; Lochter et al., 1998; Lukashev and Werb, 1998; Thomasset et al., 1998).

ORCHESTRATED EXPRESSION OF ECM-DEGRADING PROTEINASES AND THEIR INHIBITORS DURING MAMMARY GLAND INVOLUTION

A variety of secreted proteinases and proteinase inhibitors are expressed during mammary development and involution (Talhouk et al., 1991, 1992; Strange et al., 1992; Lund et al., 1995). Among the proteinases are stromelysin-1, stromelysin-3, collagenase-3, MT1-MMP, and gelatinase A (Talhouk et al., 1991, 1992; Lefebvre et al., 1992; Strange et al., 1992; Lund et al., 1995). TIMPs 1–4 are also expressed (Talhouk et al., 1992; Fata et al., 1999). The MMPs are suppressed during lactation, compared with pregnancy, but are highly expressed during involution. When extracts from involuting mammary glands are analyzed for expression of ECM-degrading proteinases, gelatinase A (72 kDa and its 62 kDa active form) and a 130 kDa gelatinase, which is not inhibited by TIMP-1, account for the major gelatinolytic species (Talhouk et al., 1991, 1992; Lund et al., 1995). Varying amounts of gelatinase B (105 kDa and its active 95 kDa form), which is made by macrophages, are also seen. The major casein-degrading enzymes with apparent molecular weights of 26 kDa, 35 kDa, 92 kDa, and >100 kDa are not secreted but instead are cell-associated. Tissue-type and urokinase-type plasminogen activator (tPA and uPA, respectively) are also found during involution of the mammary gland (outlined in Table 1). The expression such ECM-degrading proteinases suggests extensive remodeling of the basement membrane during mammary gland involution.

The transition from a fully functional lactating gland to an involuting gland is characterized by three major events. First, during lactation and the early stages of involution, ECM-degrading proteinases are expressed at low levels. MT1-MMP and gelatinase A are seen at this time (Talhouk et al., 1991, 1992; Lund et al., 1995). However, 3–4 days after weaning, stromelysin-1, stromelysin-3, tPA, uPA, and gelatinase A are upregulated (Table 1) (Talhouk et al., 1991, 1992; LeFebvre et al., 1992; Strange et al., 1992; Lund et al., 1995). This expression reaches a maximum around day 5–6 and remains high for at least 10 days into involution. Second, the expression of the proteinase inhibitors, TIMP-1 and plasminogen activator inhibitor-1, declines prior to the expression of the ECM-degrading proteinases. TIMP-1 mRNA is detected on day 2 of involution, peaks, then declines very rapidly. Third, the expression of β-casein is dramatically reduced when the balance of

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TABLE 1. Expression of Proteinases During Mouse Mammary Involution

Proteinase	Expression pattern	Reference
Stromelysin-1	Involution day 4–10	Talhouk et al., 1992 Strange et al., 1992 Lund et al., 1995
Stromelysin-3	Involution day 3–20	Lefebvre et al., 1992
Gelatinase A	Involution day 1–10	Talhouk et al., 1992 Lund et al., 1995
tPA	Involution day 3–10	Talhouk et al., 1992
uPA	Involution day 3–10	Strange et al., 1992 Lund et al., 1995
MT1-MMP	Involution day 1–10	Lund et al., 1995

ECM-degrading proteinases and their inhibitors favors the proteinases. Between days 4 and 8 of involution, the epithelial cells of the mammary gland undergo massive programmed cell death or apoptosis (Lund et al., 1995) by a mechanism involving caspases (Lund et al., 1995; Will et al., 1996) and are phagocytosed by neighboring epithelial cells or macrophages. Inhibition of MMP synthesis by treatment with pharmacologic doses of glucocorticoids in vivo completely inhibits ECM remodeling and mammary epithelial apoptosis (Lund et al., 1995). The degradation of the ECM appears to involve cooperation between epithelial and mesenchymal cells (Dickson and Warburton, 1992; End et al., 1992; Lefebvre et al., 1992; Jones et al., 1995; Lund et al., 1995). Also during this time, the adipocytes [differentiate to] fill the gland, returning it to a state similar to that of the virgin gland. These events strongly suggest that the high levels of ECM-degrading proteinases during involution result in the disruption of cell-ECM interactions that modulate mammary morphology and function.

Direct evidence for the role of MMPs and basement membrane degradation in regulating apoptosis has been found by using mouse mammary epithelial cells (MMEC) in culture (Will et al., 1996). The gelatinases are vectorially secreted in the direction of the basement membrane by MMEC, suggesting their involvement in the ECM-remodeling events of involution in vivo. In MMEC in culture, the apoptotic program involves loss of ECM, triggering the expression and activation of caspases (Boudreau et al., 1995). Similarly, apoptosis of other cell types, including endothelial cells and keratinocytes, is regulated by interactions with the ECM (Meredith et al., 1993). MMPs may alter integrin-ECM interactions to promote differentiation in this system. Different integrins may be involved in differentiation, migration, and regulation of MMPs. In mammary carcinoma cells, $\alpha 1$ and $\alpha 2$ integrins specifically regulate stromelysin-1 gene expression (Lochter et al., 1999). Taken together, these data indicate that the balance between proteinases and their inhibitors regulates integrin-ECM contacts by controlling turnover of the basement membrane. Signaling through integrins appears to be necessary for control of tissue-specific gene expression and cell viability.

TIMP EXPRESSION ALTERS ECM REMODELING

Down-regulation of epithelial-derived TIMP-1 in transgenic mice resulted in an increase in mammary ductal formation and luminal epithelial proliferation (Fata et al., 1999). The basement membrane surrounding the ducts appeared discontinuous as observed with laminin immunostaining (Fata et al., 1999). In contrast, ductal expansion was inhibited in the presence of recombinant TIMP-1-releasing pellets implanted into the mammary glands of virgin mice (Fata et al., 1999). These results suggest that perturbation of the ECM via alteration of TIMP expression affects mammary branching morphogenesis.

To probe the correlation between excess degradative activity and repression of β-casein expression, Talhouk et al. (1992) perturbed the balance directly. When slow-release pellets containing TIMP-1 were implanted into mammary glands in vivo two days after the cessation of suckling, TIMP-1 delayed both the onset of involution and the decline of β-casein expression in a dose-dependent manner. All of the MMPs detected in involuting mammary glands, with the exception of MT1-MMP (Boudreau et al., 1995), are inhibited by TIMP-1. The effect of TIMP-1 is seen not only in the stability of the basement membranes in treated mammary glands, but also in the failure of the mammary epithelial in these glands to be lost by apoptosis (Talhouk et al., 1992), suggesting that TIMPs can delay or regulate apoptosis by maintaining the integrity of the basement membrane.

MODIFIED DEVELOPMENT AND INVOLUTION IN MAMMARY GLANDS OF TRANSGENIC MICE WITH ALTERED MATRIX METALLOPROTEINASE FUNCTION

To demonstrate the importance of the ECM in promoting and maintaining tissue-specific morphology and function in vivo, we generated transgenic mice that ectopically express autoactivating isoforms of stromelysin-1 (Sympson et al., 1994, 1995). Examination of 70-day old virgin mammary glands from stromelysin-1 transgenic mice show precocious alveolar development and maturation, with primary and secondary ducts that filled the fat pad. This phenotype is both morphologically and functionally (as measured by their ability to express β-casein) very similar to that of a normal 9–12-day pregnant gland, in which endogenous stromelysin-1 is normally expressed. These results suggest that the morphology of the developing gland is exquisitely sensitive to pericellular proteolysis. Therefore, ectopic expression of stromelysin-1 appears to stimulate epithelial cell growth and differentiation in the virgin gland, producing a phenotype that resembles early pregnancy. This suggests that stromelysin-1 targeted at mammary ducts plays a role in branching morphogenesis stimulating proliferation and selection of branch points. Indeed, stromelysin-1 null mice show defective branching during puberty (unpublished

observations). Furthermore, when the CID-9 mammary epithelial cell line is transfected with the autoactivating mutant of stromelysin-1 driven by an inducible promoter, the basement membrane is disrupted, and apoptosis is triggered in the cells by a mechanism involving caspases (Boudreau et al., 1995). The precocious apoptotic phenotype can be completely reversed by crossing the stromelysin-1 transgenic mice with transgenic mice overexpressing human TIMP-1 (Alexander et al., 1996, 1999) suggesting that proteolysis regulates mammary gland morphogenesis. Apoptosis ceased in the stromelysin-1 transgenic mammary glands during lactation, even though basement membrane destruction is maximal (Alexander et al., 1996). The withdrawal of cells from the cell cycle may eliminate their sensitivity to ECM-directed apoptosis. Mammary epithelial cells in culture are in the cell cycle before initiating apoptosis (Boudreau et al., 1996). [In support of this model our data indicate that] during involution [after weaning], mouse mammary epithelial cells reenter the cell cycle in a very narrow window of 36 to 48 hr after initiation of weaning, before undergoing apoptosis (Fig. 1, Wiesen et al., submitted), as has been seen in the bovine mammary gland (Capuco et al., 1997). This time parallels initiation of the first proteinase-independent phase of involution (Lund et al., 1995).

CELL CYCLE REGULATION AND APOPTOSIS DURING MAMMARY GLAND INVOLUTION

In the involuting gland, apoptosis starts at 2 days, and peaks at 4 days postweaning and is largely confined to the epithelial cells that line the alveoli (Lund et al., 1995). The increase in the number of cells entering S phase at 36 hr is largely confined to the epithelial cells of the alveoli (Fig. 1). Cell proliferation occurs not only during the ductal growth and branching seen during virgin development, and during the lobulo-alveolar development of pregnancy, but also during mammary involution. There is a peak of [cell proliferation] DNA synthesis at 48 hr of involution (Wiesen et al., submitted). Similarly, precocious apoptosis occurs in the stromelysin-1 transgenic mice during pregnancy, when the epithelial cells are actively proliferating, ceases during lactation, when these cells exit the cell cycle (Thomasset et al., 1998; Alexander et al., 1999). The proteolysis or removal of an appropriate extracellular matrix in culture also induces apoptosis of MMEC (Boudreau et al., 1995, 1996). Induction of proliferation during the radical changes in the cellular environment that accompany involution results in conflicting signals that also induce expression of $p21^{cipl}$ and c-myc (Boudreau et al., 1996). Such misexpression of cell cycle regulatory components leads to apoptosis of the epithelial cells. If c-myc is overexpressed in MMEC or fibroblasts during serum deprivation, apoptosis occurs (Evan et al., 1992; Fowlkes et al., 1995). In transgenic mice, overexpression of Bcl-2 inhibits mammary apoptosis and accelerates c-myc-induced tumorigenesis (Jager et al., 1997). Therefore, the extracellular matrix, along with hormones and growth factors, contribute signals to the cell cycle machinery that modulate the level of cyclin expression in the G1 phase of the cell cycle. It is the combination of these signals that determine whether the cell progresses through the G1/S restriction point or undergoes apoptosis.

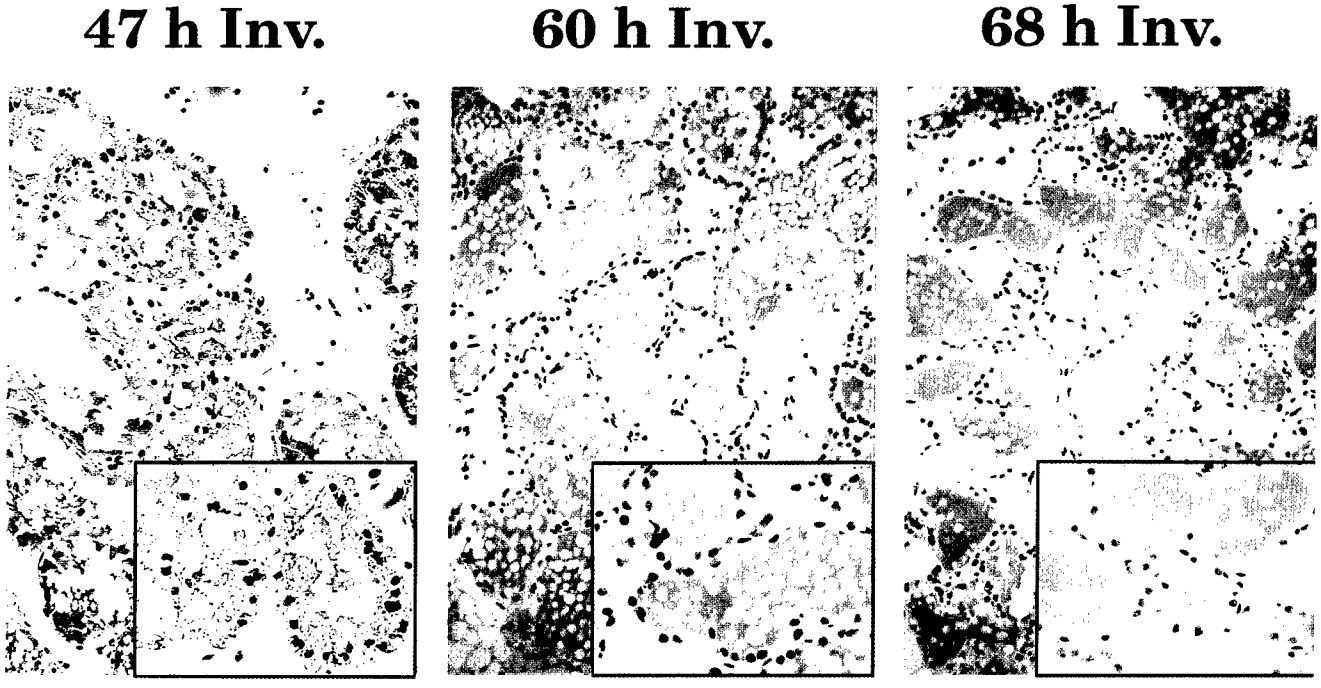

Fig. 1. Mammary epithelial cells enter the cell cycle prior to the onset of apoptosis. A wave of cell proliferation, as determined by BrdU incorporation occurred at 36–48 hr of involution, and approximately 95% of these proliferating cells underwent apoptosis within 12–18 hr. Glands from mice injected with BrdU at 46 hr of involution (Inv.) and killed 1, 14, and 22 hr later are shown. All panels are at 200× magnification, and the insets are at 400× magnification (modified from Wiesen et al.).

There are several clues to the mechanisms underlying the induction of apoptosis. First, like MMPs, interference with β1 integrins in culture (Boudreau et al., 1995, 1996) and in vivo (Faraldo et al., 1998) leads to apoptosis (reviewed by Streuli and Gilmore, 1999). Secondly, β1 integrins regulate cell cycle progression and proliferation (Assoian, 1997). The initiation of apoptosis has been correlated in several cell types with aberrant regulation of the cell cycle and proliferation. Analysis of a variety of cells in tissue culture has shown that at least one of the cyclins is induced in the absence of the rest of the normal regulatory pathway for proliferation with the timing of induction that associates it closely with commitment to apoptosis (Hoang et al., 1994; Shi et al., 1994; Lochter et al., 1997). Inhibiting the induction of cyclins or the activation of associated kinases suppresses apoptosis (Shi et al., 1994). Suppression of apoptosis by stable overexpression of bcl-2 prevents the inappropriate expression or activation of the cell cycle regulatory molecules (Hoang et al., 1994; Shi et al., 1994), in one case by allowing induction of the normal complement of cyclins (Hoang et al., 1994). The specific cell cycle regulatory molecules that are induced during apoptosis differ between cell types, e.g., cyclin D1 in cultured MMEC (Boudreau et al., 1996), cyclin A in embryonic fibroblasts (Hoang et al., 1994) and B-type cyclins in hematopoietic cells (Shi et al., 1994). In mammary epithelium cyclin D1 is expressed during apoptosis (Fowlkes et al., 1995). It is not yet clear whether misregulation of the cell cycle is a necessary component of apoptosis in all cases, as there are several examples in which there are as yet no obvious aberrations in cell cycle regulation (Furuya et al., 1995). The observations that link apoptosis and misregulation of the cell cycle are just beginning to be extended into intact tissues.

CELL CYCLE CONTROL IN MAMMARY GLAND DEVELOPMENT AND CANCER

In mammalian cells, progression through the cell cycle is regulated by cyclin-dependent kinases (CDKs), which act sequentially with the cyclins to modulate transitions through the cell cycle checkpoints (Murray and Hunt, 1993; Said and Medina, 1995). Control of mammalian cell proliferation by extracellular signals occurs primarily during the G1 phase of the cell cycle. Key regulators of cell cycle progression include the D-type cyclins, which associate with CDK4 and CDK6, and cyclin E, which associates with CDK2 later in G1 (Sherr, 1994). D-type cyclins are synthesized in response to growth factor stimulation and are rapidly degraded when the mitogenic signal is removed regardless of the position of the cell in the cycle (Sherr, 1994). The ability of the D-cyclins to act as growth factor sensors links the extracellular environment to the core of the cell cycle machinery.

Due to the critical role that the cyclins play in regulating cell proliferation in response to extracellular signals, deregulation of these cyclins would make cell cycle progression less dependent on growth factors and could lead to oncogenesis (Hunter and Pines, 1994). Cell cycle components that are known to be altered during oncogenesis are indicated in Figure 2 and include cyclins A, E, and D1, 2, and 3. Misregulation of the D-type cyclins could result in the cell cycle machinery sensing a state usually induced by growth factors and cause the cells to proliferate rather than differentiate, fulfilling one of the conditions necessary for cellular transformation (Hunter and Pines, 1994). If epithelial cells are the target of aberrant cyclin D1 regulation, then proliferation may result in the absence of growth factors, rather than apoptosis, which is seen in secretory alveolar epithelium in the absence of growth factors (Fowlkes et al., 1995). Indeed, cyclin D1 is a commonly overexpressed oncogene, seen in 50% of breast cancers (Bartkova et al., 1994). When cyclin D1 is overexpressed in the mammary glands of transgenic mice, abnormal cell proliferation and the development of adenocarcinomas result (Wang et al., 1994). Cyclin D1 is normally expressed in the mammary epithelium during the lobulo-alveolar development associated with pregnancy (Sicinski and Weinberg, 1997). Both estrogen and progesterone induce cyclin D1 expression in vivo (Said et al., 1997). In mice lacking cyclin D1, lobulo-alveolar development fails to occur, while ductal development is normal (Fantl et al., 1995; Sicinski et al., 1995). However, overexpression of cyclin D1 in MMEC in culture inhibits growth and induces apoptosis (Boudreau et al., 1996; Han et al., 1996). Similarly, when regulators of CDKs, such as the cdc 25 phosphatases are overexpressed in the mammary gland, an increase in cyclin D1 protein is seen during the resulting alveolar hyperplasia (Ma et al., 1999; Yao et al., 1999). When cyclin E is overexpressed in mouse mammary glands, lactating glands contain areas of hyperplasia, and mammary carcinomas develop (Bortner and Rosenberg, 1997). Cyclin A also appears to be involved in apoptosis (Hoang et al., 1994). The mammary glands of transgenic mice overexpressing cyclin A contain nuclear abnormalities, increased apoptosis, and areas of hyperplasia (Bortner and Rosenberg, 1995). By contrast, mice deficient in Lats 1, a putative serine/threonine kinase that functions as a negative regulator of the activity of the CDK1 (CDC2) complex with cyclin A, have reduced mammary ductal epithelium and some animals, no nipples and only mammary fat pads without any epithelial ductal components (St. John et al., 1999). These observations are summarized in Table 2. Together, these mouse models reveal the importance of maintaining the correct level of cyclin proteins necessary for normal development of the mammary gland and show how misregulation of these cell cycle proteins can lead to defective mammary gland development, hyperplasia, and adenocarcinomas.

SUMMARY

These studies clearly demonstrate that coordinated expression and a critical balance between ECM-degrading proteinases and their inhibitors regulate

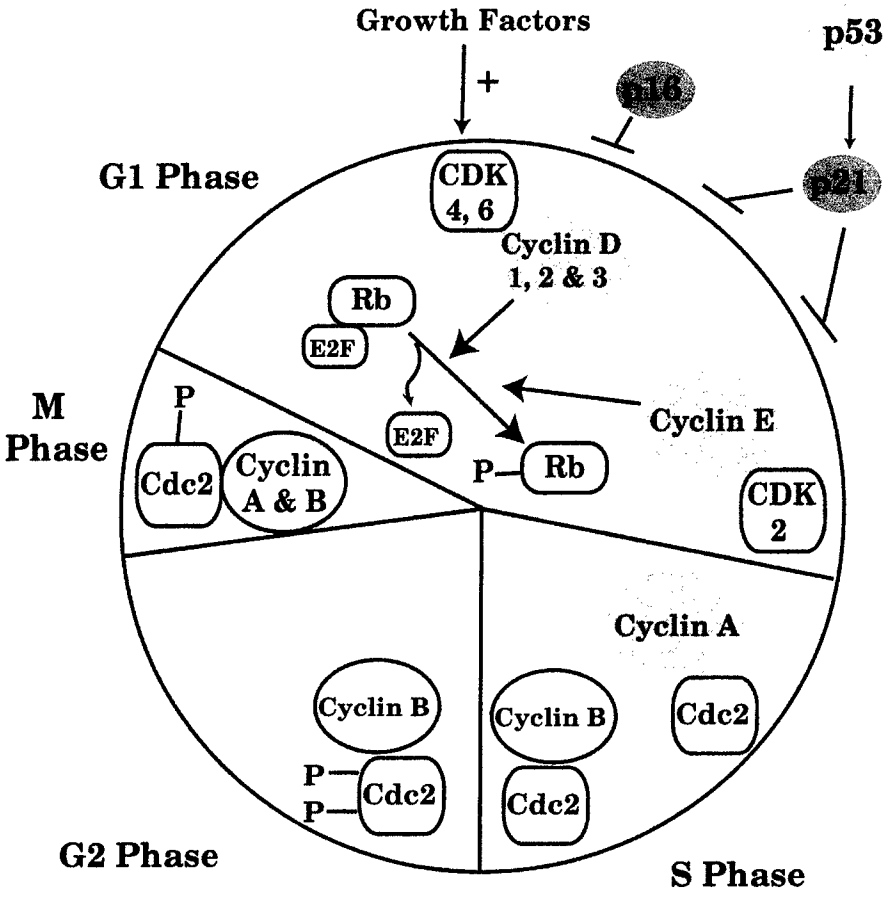

Fig. 2. Cell cycle regulators implicated in cancer. The components of the cell cycle that are frequently mutated or overexpressed in tumors are lightly shaded (e.g., cyclin D) and those regulators that are often missing in tumors (e.g., p21) are darkly shaded.

TABLE 2. Mammary Gland Phenotypes in Mice With Transgenic Alteration of Cyclin Gene Expression

Gene[1]	Mammary phenotype	Reference
Cyclin D1$^{-/-}$	No lobular-alveolar development Ductal development is normal	Sicinski et al., 1995, 1997 Fantl et al., 1995
MMTV-cyclin D1	Adenocarcinoma	Wang et al., 1994
BLG-cyclin E	Hyperplasia Adenocarcinoma	Bortner and Rosenberg, 1997
BLG-cyclin A	Nuclear abnormalities Increased apoptosis Hyperplasia	Bortner and Rosenberg, 1995
Lats 1	No epithelial component in mammary gland	St. John et al., 1999

[1]MMTV, mouse mammary tumor virus; BLG, ovine β-lactoglobulin.

epithelial cell proliferation, function, and apoptosis during development of the mammary gland. As the ratio of ECM-degrading proteinases to inhibitors increases, basement membrane is degraded, secretory mammary epithelial cells lose their ability to express β-casein and undergo apoptosis, and alveolar regression becomes evident. A model for the series of events that occur during, and regulate the process of, involution can now be drawn from in vivo and culture studies of mouse mammary glands. After cessation of milk removal from the gland, milk continues to be secreted and accumulates in the alveolar lumina for 1–2 days. At this time, local factors induce the expression of cell cycle regulatory genes, ECM-degrading proteinases, and their inhibitors in a coordinated and temporal pattern. The events that regulate cell-ECM interactions occur in a microenvironment at the individual cell level within an alveolus. Each cell or group of cells within an alveolus is part of a milieu with various amounts of either the ECM-degrading proteinases or the inhibitors. The ECM-degrading proteinases and their inhibitors are secreted either by the epithelia

themselves or by neighboring fibroblasts in the underlying stroma. The postulated events that occur at the level of an individual alveolus are as follows. As the lumen of a particular alveolus is engorged owing to cessation of milk removal, perhaps a local signal instructs certain cells in the alveolus to enter the cell cycle (Wiesen et al., submitted; Capuco et al., 1997), while other cells are triggered in the immediate microenvironment (myoepithelial cells or fibroblasts) to secrete ECM-degrading proteinases (Sympson et al., 1994; Witty et al., 1995). Local concentrations of ECM-degrading proteinases then degrade the basement membrane in the immediate vicinity and alter cell-ECM interactions. This leads to the generation of bioactive ECM fragments and eventually to detachment of a cell from its degraded basement membrane and its neighbors. At some time during this process the cells lose their ability to express milk proteins. The detachment of the cells from the basement membrane also triggers expression of cell cycle inhibitors. These conflicting signals induce the apoptotic cell death program, including the induction of caspase gene expression. Apoptotic events increase as the ratio of ECM-degrading proteinases to inhibitors increases during involution of the mammary gland, or in an unscheduled way in the glands from midpregnant stromelysin-1 transgenic mice. After the apoptotic cell detaches from its underlying degraded basement membrane and from neighboring cells in the alveolus, the remaining cells, which rest on an intact basement membrane, join. As involution proceeds, the net result is first a smaller alveolus with a continuous basement membrane that is then obliterated (Talhouk, et al., 1992). It is not clear what regulates these events at the individual alveolus, and why certain cells within an alveolus are destined to die before others. It is possible that only cells that are progressing through the cell cycle are able to die. The local concentrations of inhibitors bound to the basement membrane protect surviving cells within an alveolus and temporally regulate programmed cell death and alveolar regression. Taken together, the observations in mammary glands in which the balance of MMPs and inhibitors has been perturbed indicate that in the context of a normal gland, ECM remodeling is both necessary and sufficient to alter the phenotype of mammary epithelial cells.

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MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF MATRIX METALLOPROTEINASES IN THE STROMA REGULATE MAMMARY DUCTAL MORPHOGENESIS.

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Mammary gland ductal morphogenesis begins in late fetal development, continues slowly during the early post-natal period and accelerates in response to ovarian hormones at the onset of puberty. It is well known that ductal epithelial cell morphogenesis is regulated by stromal factors in the surrounding fat pad. Here we show that stromal matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), influence ductal elongation and branching by regulating extracellular matrix remodeling. Transgenic mice that constitutively over-express TIMP-1 show diminished ductal elongation and branching, and branching of cultured mammary organoids is blocked by synthetic MMP inhibitors in vitro. However, these models do not indicate which MMPs are responsible for ductal elongation and branching. MMP-3/stromelysin-1 and MMP-2/gelatinase A are both upregulated at the mRNA and protein levels in the stroma surrounding the developing ducts making them particularly good candidates. In order to determine which MMPs are involved in ductal elongation and branching we have examined mammary gland development in specific MMP over-expressing and null mouse models. MMP-3 has no effect on ductal penetration but its over-expression increases, and its absence significantly decreases, both dichotomous and lateral branching. On the other hand, the MMP-2 null mice exhibit reduced ductal penetration of the fat pad without changes in branching. MMP-9/gelatinase B is not normally upregulated at puberty and, not surprisingly, its absence in MMP-9 null mice has no effect on ductal development. The transmembrane MMP, MMP-14, regulates MMP-2 activation at the cell surface. Consequently, deletion of the MMP-14 protease from the mammary gland may also inhibit ductal development through regulation of MMP-2 activation. These animals exhibit retarded adipocyte differentiation within the mammary fat pad and we are currently evaluating whether epithelial alterations exist as well. Although we have shown that MMP-2 and MMP-3 have distinct roles during ductal development, the absence of either MMP-2 or MMP-3 fails to ablate ductal morphogenesis entirely indicating that other MMP family members are likely to have a role in these processes.

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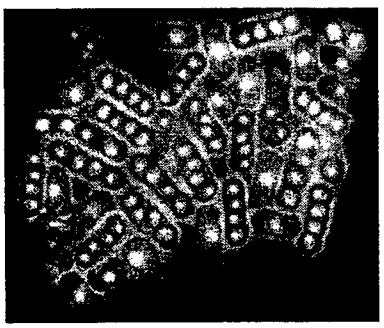
Meiotic machinations

DNA replication is a central event in each mitotic cell cycle, and the factors involved in this process are coming into finer focus. For example, it was recently established that the conserved mini-chromosome maintenance (MCM) proteins help to initiate replication by assembling a pre-replicative complex that mediates origin firing during S phase[1].

DNA replication is also a critical prelude to chromosome segregation during meiosis, but less is known of its molecular players. The naïve assumption would be that analogous events of mitotic and premeiotic DNA replication are coordinated by the same molecules. On page 263, however, Susan Forsburg and Jeffrey Hodson[2] trounce this assumption. Upon screening mutants with defective mitotic DNA replication, they discovered that a group of initiation genes essential to replication in vegetative cells (including *mcm2*$^+$ and *mcm4*$^+$) are dispensable during meiosis. In contrast, elongation factors, such as DNA



polymerases and DNA ligase, are required for premeiotic S phase—just as they are for mitotic DNA replication. These surprising results indicate that mitotic initiation factors have no role in premeiotic DNA replication, or, alternatively, are rendered redundant by one or more overlapping meiosis-specific pathways.

It would seem that meiosis has evolved from mitosis and, in so doing, imposes new constraints on mitotic processes. For example, double-strand break-repair proteins that repair damage during mitosis have been co-opted by meiosis-specific proteins, so that the choice recombination partner—that is, homologous chromosome rather than sister chromatid—serves the specialized needs of haploid gamete formation[3]. Whether the observations of Forsburg and Hodson are due to a similar imposition of meiosis-specific constraints upon the mitotic replication machinery remains to be determined. In any case, the lesson is clear: when it comes to extrapolating from mitotic events to their meiotic counterparts, check your assumptions at the door. —*Scott Keeney*

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The labyrinthine placenta

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Fetal nutrition depends on the placenta, and specifically, the branching of a layer of placental trophoblast cells that sits between the maternal blood and fetal blood vessels. The discovery that expression of the transcription factor Gcm1 in trophoblast stem cells regulates branching of this specialized epithelium offers new insights into a process whose pivotal features have been generally obscure.

The formation of a proper placenta is key to mammalian fetal development: without the placenta, fetuses die even in the absence of obvious defects[1]. On page 311 of this issue, Lynn Anson-Cartwright and colleagues[2] report that the branching that generates the labyrinthine structure at the centre of the placenta is regulated by *Gcm1*, which encodes the transcription factor glial cells missing-1. Anson-Cartwright *et al.* and Jorg Schreiber *et al.*[3] (who have recently reported a similar study in *Molecular Cellular Biology*) found that mice lacking *Gcm1* fail to form the labyrinthine layer and succumb to placental insufficiency.

The placenta starts to form at a time when the metabolic requirements of the growing mouse embryo approach the capacity of the yolk sac. It is formed from the fusion of two tissues: the allantois, which derives from extra-embryonic mesoderm and eventually develops into the umbilical cord, and the extra-embryonic chorion (Fig. 1*a*), which derives from the polar trophectoderm overlying the inner cell mass of the blastula. Fetal blood vessels grow out of the allantoic mesoderm, invade the chorionic plate and instruct the trophoblast stem cells that reside within the chorionic plate to differentiate (Fig. 1*b*,*c*). At this point, the trophoblast cells fuse, forming syncytiotrophoblasts, and the three layers (allantoic mesoderm, interstitial syncytiotrophoblasts and chorion) come together to form the haemotrichorial labyrinth. Integration of fetal and maternal blood vessels within the labyrinth is critically dependent on the differentiation of the trophoblast stem cells to syncytiotrophoblasts. As the labyrinth forms, the chorion gives rise to trophoblast stem cells of the ectoplacental cone. Trophoblast giant cells differentiate from these stem cells and migrate outwards, embedding themselves in the uterine wall to anchor the forming placenta.

Despite superficial differences, the labyrinth in the mouse and the floating chorionic villi in human are homologous structures, both characterized by extensive branching. And the similarities seem unlikely to stop there. The development of both mouse and human placentae (Fig. 2) may be partly regulated by the Gcm and basic helix-loop-helix (bHLH) gene families. In the mouse, two bHLH family members, Hand1 and Mash2, have antagonistic actions in determining trophoblast cell fate. Whereas Mash2 maintains trophoblast stem cells, Hand1 promotes the differentiation of tro-

phoblast giant cells[1,4]. The activity of each protein is regulated through its interaction with common dimerization partners; their success at competing for these factors determines whether differentiation takes place[4]. *HAND1* is not expressed in the human placenta[5], but other members of the bHLH family may have an equivalent role to mouse *Hand1*. *HASH2*, however, an orthologue of *Mash2*, is highly expressed in cytotrophoblast cells throughout pregnancy, as is *GCM1* (which is not a bHLH protein).

Another bHLH family member at work in the placenta is *ID2*, whose expression is strong in cytotrophoblast stem cells but dissipates as they differentiate and invade the maternal blood vessels. Overexpression of *ID2* in cytotrophoblast cultures increases the expression of *GCM1*, indicating that members of the GCM and bHLH families coordinately regulate cytotrophoblast differentiation[5]. The placentae of women with severe cases of pre-eclampsia, a disorder that sets in during the first trimester and involves hypertension, proteinuria and oedema, show reduced branching of the chorionic villi[6] which may be caused by an abnormally low activity of a GCM or bHLH family member.

Mouse mutations

A vital characteristic of the haemotrichorial layer—that it facilitates and permits nutrient and gaseous exchange between mother and fetus—is consistent with runting or fetal death caused by mutations that affect it. In addition to the *Gcm1* mutation, several other mouse mutations have been described that block distinct steps of placental development[1,3,7-10], in the absence of obvious embryonic defects. For example, in mice deficient in Hsp90β (a 'chaperone' protein that mediates protein folding), fetal vessels invade the chorion[7] but trophoblast stem cells do not differentiate into syncytiotrophoblasts—and so a labyrinth never forms. Complementation with wild-type allantoic mesoderm, however, rescues labyrinth formation, indicating that Hsp90β is essential to syncytiotrophoblast differentiation.

In a similar manner to *Gcm1* ablation, Pparγ deficiency[8] has a generally negative effect on the formation of a functional epithelial barrier by the syncytiotrophoblasts in the haemotrichorial layer. *Gcm1* may be upstream of *Pparg*, as some branching and formation of some trilaminar layers is observed in the *Pparg*$^{-/-}$ embryos. Alternatively or concomitantly, mutant Pparγ may affect syncytiotrophoblast differentiation independently, as trophoblasts at the haemotrichorial interface are unusually thick and accumulate lipids.

Branching out

When you mention the term "epithelial branching morphogenesis" in casual conversation (as one does), most people think: heart, breast, lung. Indeed, it is from these tissues that we have learned a great deal about the way in which interactions between the mesenchyme and epithelium direct and regulate branching morphogenesis. These tissues have provided us with several gene families that either signal or regulate branching morphogenesis[11]. For example, a transcription factor essential to branching morphogenesis of the embryonic kidney and lung is *Pod1*, another member of the bHLH family. Deletion of this gene results in perinatal lethality due to failure to form terminal air sacs, alveoli, and the air-ducts that supply them—all defects in terminal branching[11]. These animals also fail to develop fully differentiated glomeruli and have reduced numbers of glomeruli overall, another failure of terminal branching. In the rare cases where terminal branching does occur, the epithelium lining the branches is not fully differentiated or changes its fate.

In the placenta, the allantoic mesoderm instructs the chorionic stem cells, which then initiate the branching program through Gcm1. Unlike *Gcm1*, which regulates the earliest branching of the labyrinthine layer, *Pod1* is expressed in mesenchyme and is dispensable for early branching in both kidney and lung. Neither *Gcm1*$^{-/-}$ trophoblasts nor *Pod1*$^{-/-}$ lung and kidney epithelia terminally differentiate but, in contrast with *Pod1*$^{-/-}$ lung epithelia, *Gcm1*$^{-/-}$ trophoblasts remain unaltered (that is, they fail to differentiate, instead of differentiating inappropriately). This indicates differences in the differentiation pathways regulated by these genes.

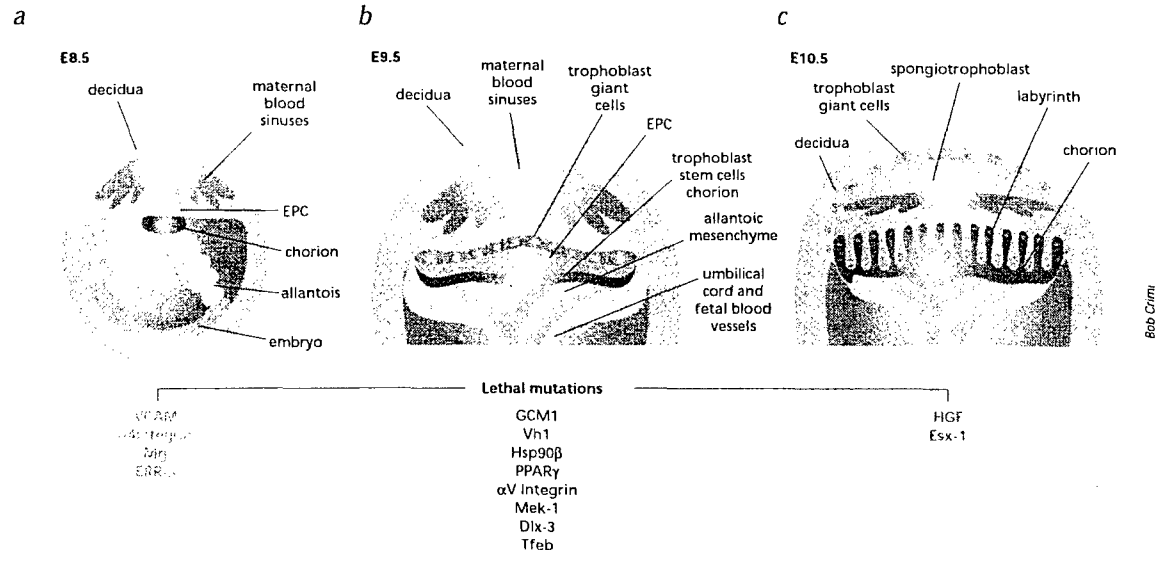

Fig. 1 Mutations affecting mouse placental development. **a**, Mutations in genes encoding V-cam, a4 integrin and MJR (a co-chaperone) result in failure of the allantois and chorion to fuse. **b**, Lethal mutations affecting development at embryonic day (E) 9.5 to 10.5 (when the placenta supercedes the yolk sac) affect trophoblast (*Gcm1, Hsp90b, Vhl, Dlx3*) or fetal vessel function (*Pparg, Tfeb, Mek1* and the gene encoding αV-integrin); all of these[1,3,7-10] confound formation of the labyrinth. **c**, *Hgf* and *Esx1* mutations (which are not always lethal) affect later labyrinth development- from E11.5 to E16.5 (ref. 1).

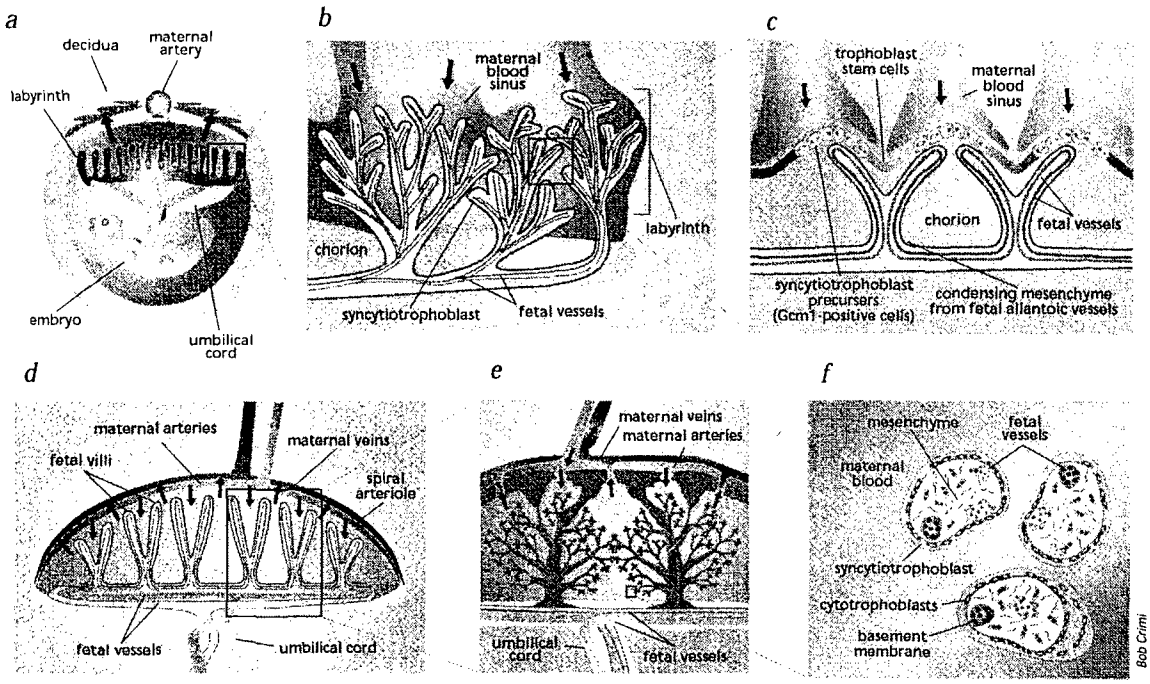

Fig. 2 Development of the mouse (*a–c*) and human (*d–f*) placenta. ***a***, At E10.5, the labyrinth forms. Maternal vessels are indicated in red, and fetal vessels, in light blue. ***b***, The fetal vessels from allantoic mesoderm penetrate the chorion and invade the labyrinth. Syncytiotrophoblast (indicated in purple) forms around the developing fetal vessels. Maternal blood sinuses also enter the labyrinth and supply blood to the fetal vessels across the syncytiotrophoblast barrier. ***c***, Trophoblast stem cells from the chorion express Gcm1 at the developing tips of the branching epithelium. Fetal mesenchyme containing vessel precursors undergoes branching morphogenesis and invasion behind the trophoblast layer. The invading trophoblasts will become the syncytiotrophoblast barrier between maternal blood sinuses and fetal blood vessels. ***d***, Human placenta showing fetal villi and umbilical cord with fetal vessels in villi. Villi are bathed in maternal blood, which exits through the maternal veins. A syncytiotrophoblast sheath around the villi prevents direct contact between maternal and fetal blood. ***e***, Two fetal villus trees illustrate the complex branching pattern. Each villus branch is also covered with branched microvilli (not shown) and surrounded by syncytiotrophoblast (indicated in purple). ***f***, Each villus, depicted in cross section, is bound by a syncytiotrophoblast layer and bathed in maternal blood. Inside the villus is a layer of cytotrophoblast and the basement membrane. The central part of the villus contains fetal mesenchyme and vessels.

Invasion of the vasculature

Studies of bone formation[12] clearly indicate that tissue morphogenesis is dependent on the development of, and coordination with, the vascular system. How this is regulated in organs like the kidney or lung remains obscure. From the work of Anson-Cartwright *et al.*[2], however, it is clear that fetal vessel invasion and interdigitation with maternal blood sinuses require chorionic trophoblast branching morphogenesis and syncytiotrophoblast differentiation. Other mouse mutations result in lack of fetal vessel development or invasion of vessels into the labyrinth. For example, mutation of *Esx1*, which has a similar expression pattern to *Gcm1*, negatively regulates the branching of fetal vessels within the labyrinth[13]. Syncytiotrophoblast differentiation and the barrier function of the haemotrichorial layer are also affected in the $Esx1^{-/-}$ mice, which are runted at birth.

Whereas some pathways are likely to be different between mouse and human, a detailed understanding of the mechanisms that regulate branching morphogenesis during placental development will shed light on how these processes are altered in two severe problems of human pregnancy, pre-eclampsia and intrauterine growth retardation. Progress in understanding branching morphogenesis in the placenta also raises many new questions. What regulates the size of the surface area across which gas and nutrient exchange takes place? What mechanisms control the number and position of bifurcations? Does branching occur in response to hypoxic signals from the fetus? Why are there distinct trophoblast subtypes involved in invasion and the maternal-fetal barrier? The recent development of trophoblast stem cell lines[14] offers promise not only for elucidating the differentiation of trophoblast lineages, but also for the rescue of fetuses with defective trophoblasts. □

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Mammary Epithelial Cells Enter the Cell Cycle Prior to the Onset of Apoptosis During Involution

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Running Title: Proliferation and Apoptosis in Mammary Epithelial Cells

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Mammary gland involution is characterized by the loss of differentiated function, and remodeling of the mammary gland via the process of apoptosis. Cell interactions with the extracellular matrix or the basement membrane are crucial to the maintenance of the differentiated phenotype, withdrawal from the cell cycle, and the suppression of apoptosis. During mammary gland involution, cell-matrix interactions are disrupted, and the epithelial cells undergo a massive wave of apoptosis. In this report, we demonstrate that mammary epithelial cells enter the cell cycle prior to the onset of apoptosis during the process of involution. Steroid hormones, growth factors, and cell-matrix interactions all contribute to determining if mammary epithelial cells are in the cell cycle, whether cell cycle progression occurs, or whether the cell undergoes apoptosis. Misregulation or overexpression of the cell cycle proteins drastically alters mammary gland development and may play a role in oncogenesis.

Keywords: Mammary gland, cell cycle, cyclins, involution, proliferation

INTRODUCTION

In the mammary gland, cell proliferation is induced by the action of the steroid hormone estrogen, which induces ductal development during puberty (1-2), and progesterone which stimulates lobulo-alveolar development necessary for lactation (3-5). The actions of these steroid hormones are mediated in part by the local actions of growth factors such as the ligands of the EGF family (6-8), HGF (9-11), and KGF (12-14). Hormonal stimulation induces the cell to enter the cell cycle and promotes progression through the cell cycle (15-16). Depending on the balance of the various mitogenic signals, the cell may undergo proliferation, differentiation, or apoptosis. If the cell receives conflicting signals, such as the expression of factors that promote cell proliferation while cell progression is blocked, then apoptosis will result. For instance, the proto-oncogene *c-myc* is an essential part of the cell proliferative machinery and can induce both cell cycle progression and apoptosis (17). However, if *c-myc* is overexpressed in fibroblasts during serum deprivation, apoptosis occurs (18). The correct tissue architecture is also required to maintain differentiation and to suppress apoptosis. Mammary epithelial cells require extracellular matrix to express the differentiated phenotype, expression of the milk protein β-casein (19-20). The presence of a basement membrane suppresses apoptosis by down-regulating the expression of *c-myc* and cyclin D1, and results in withdrawal of the mammary epithelial cells from the cell cycle (21). During mammary gland involution, it is the loss of this basement membrane that induces the epithelial cells to undergo apoptosis, and in this review we will demonstrate that these cells first enter the cell cycle prior to the onset of apoptosis.

Mammalian Cell Cycle Progression

In mammalian cells, progression through the cell cycle is regulated by cyclin-dependent kinases (CDKs), which act sequentially with the cyclins to modulate transitions through the cell cycle checkpoints (22). Control of mammalian cell proliferation by extracellular signals occurs primarily during the G1 phase of the cell cycle. Key regulators of cell cycle progression include the D-type cyclins (D1, D2, and D3) which associate with CDK 4 and CDK6 and cyclin E, which associates with CDK2 later in G1 (23). D-type cyclins are synthesized in response to growth factor stimulation and are rapidly degraded when the mitogenic signal is removed regardless of the position of the cell in the cycle (23). The ability of the D-cyclins to act as growth factor sensors links the extracellular environment to the core of the cell cycle machinery.

Cell Cycle Control and Cancer

Due to the critical role that the D-type cyclins play in regulating cell proliferation in response to extracellular signals, deregulation of these cyclins would make cell cycle progression less dependent on growth factors and lead to oncogenesis (24). Misregulation of the D-type cyclins would result in the cell cycle machinery sensing that the growth factors are constantly present and cause the cells to proliferate rather than differentiate, fulfilling one of the conditions necessary for cellular transformation (24). While a number of cyclins are overexpressed in human cancers, cyclin D1 is a commonly overexpressed oncogene, seen in 50% of breast cancers (21, 25). Overexpression of cyclin D1 in the mammary glands of transgenic mice results in abnormal cell proliferation and the development of adenocarcinomas (26). Cyclin D1 is normally expressed in the mammary epithelium during the lobulo-alveolar development associated with pregnancy (27). In mice lacking cyclin D1, lobulo-alveolar development fails to occur, while ductal development is normal (28-29). Outlined in Table I are the mammary phenotypes seen when specific cyclins are overexpressed or removed. When cyclin E was overexpressed in mouse mammary glands, lactating glands contained areas of hyperplasia, and mammary carcinomas developed (30). While transgenic mice which overexpressed cyclin A contained nuclear abnormalities, increased apoptosis, and areas of hyperplasia (31). Together, these mouse models reveal the importance of having the correct level of cyclin proteins necessary for normal development of the mammary gland and how misregulation of these cell cycle proteins can lead to hyperplasias, and adenocarcinomas.

Proliferation and Apoptosis During Mammary Gland Involution

Following the cessation of lactation, the mammary gland undergoes involution, a remodeling event that is initiated by the proteolytic degradation of the basement membrane and results in a large wave of apoptosis (32). During this process of involution the expression of milk protein genes fall, the alveolar structures collapse, the secretory luminal epithelial cells, the endothelial cells, and the myoepithelial cells are removed by apoptosis and replaced by adipose tissue (33-35). In the mouse mammary gland, this wave of apoptosis begins on day 2 of involution with the maximal rate of cell death occurring on day 4 of involution (33). A wave of cell proliferation precedes this wave of apoptosis, (Wiesen and Werb, unpublished). As seen in Figure 1, cell proliferation as measured by bromodeoxyuridine (BrdU, 300 μg/mouse) incorporation, occurs not only during the ductal growth and branching seen during virgin development, and during the lobulo-alveolar development of pregnancy, but also during mammary involution. There is a peak of cell proliferation at day 2 of involution (Wiesen and Werb, unpublished). To determine the relationship between proliferation and the onset of apoptosis in the mammary epithelial cells, pulse-chase experiments were performed in which BrdU was administered starting at 24 hours of involution, and re-administered every 6 hours for 24 hours to label the entire population of proliferating cells. These experiments reveal that of the luminal epithelial cells that are proliferating and therefore incorporating BrdU, approximately 95% of these cells undergo apoptosis within 12-18 hours. While many mammary epithelial cells are labeled at 47 hours of

involution, 13 hours later (at 60 hours of involution) few labeled cells remain, and almost all labeled cells are lost 21 hours later (at 68 hours of involution) as seen in Figure 2. This demonstrates that the mammary epithelial cells entered the cell cycle prior to the onset of apoptosis. Additional experiments are underway to determine whether the onset of apoptosis would be affected by blocking cell cycle progression. Interestingly, parallel experiments which examined apoptosis during uterine regression revealed that in contrast to the mammary epithelial cells, uterine epithelial cells do not enter the cell cycle prior to the onset of apoptosis (Wiesen and Werb, unpublished). Cell interactions with the extracellular matrix suppress apoptosis by down-regulation of cell cycle proteins and withdrawal from the cell cycle (21). oOur results indicate that, when the interaction of the cells with the basement membrane is disrupted, the epithelial cells enter the cell cycle, where the decision is made at the restriction checkpoint of whether to continue proliferating or to undergo cell death. In the case of mammary gland involution, those cells in the cell cycle no longer receive the needed proliferative signals via growth factor stimulation or contact with the extracellular matrix and therefore apoptosis ensues. In summary, hormones, growth factors, and the extracellular matrix contribute signals to the cell cycle machinery that modulate the level of cyclin expression in the G1 phase of the cell cycle. It is the combination of these signals which determine whether the cell progresses through the G1/S restriction point or undergoes apoptosis.

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Table 1. Mammary Gland Phenotypes in Transgenic and Cyclin Null Mice.

Cyclin:	Mammary Phenotype:	Reference:
D1 $^{-/-}$	No lobular-alveolar development Ductal development is normal	Sicinski et. al Fantl et. al
MMTV-D1	Adenocarcinoma	Wang et. al
BLG-E	Hyperplasia Adenocarcinoma	Bortner and Rosenberg[30]
BLG-A	Nuclear abnormalities Increased apoptosis Hyperplasia	Bortner and Rosenberg[31]

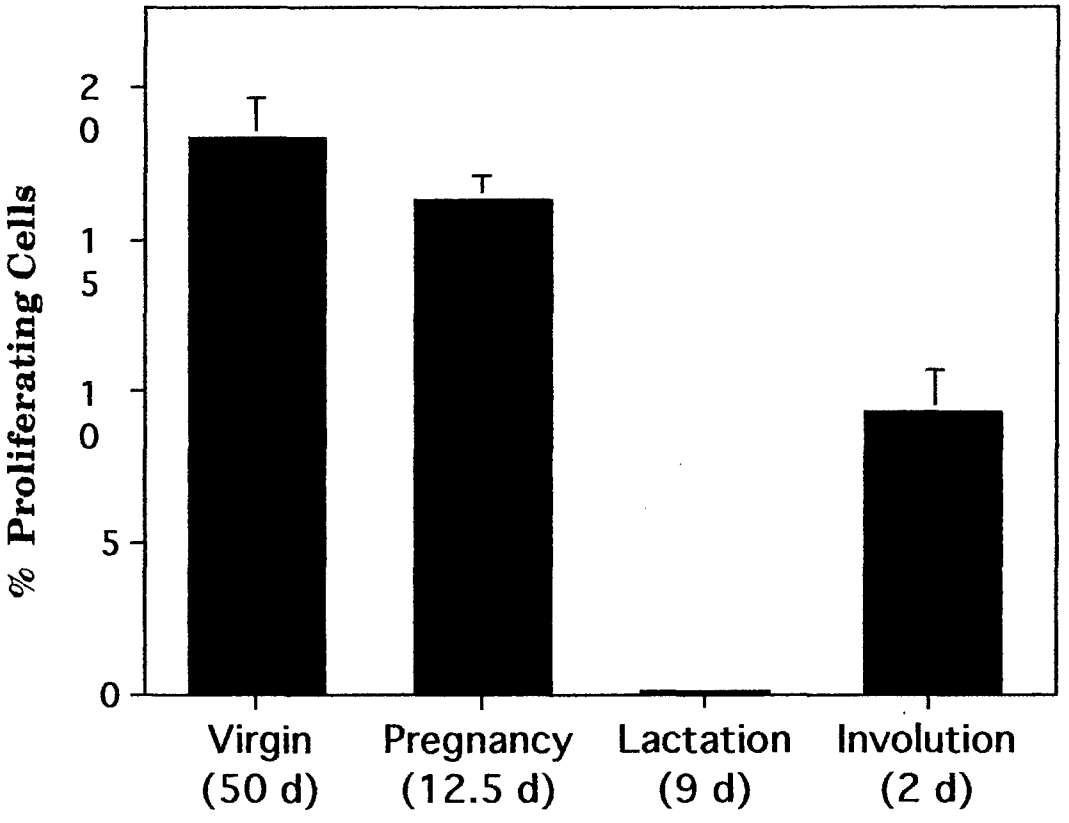
Mouse Mammary Tumor Virus (MMTV), Ovine Beta-lactoglobulin (BLG)

FIGURE LEGENDS

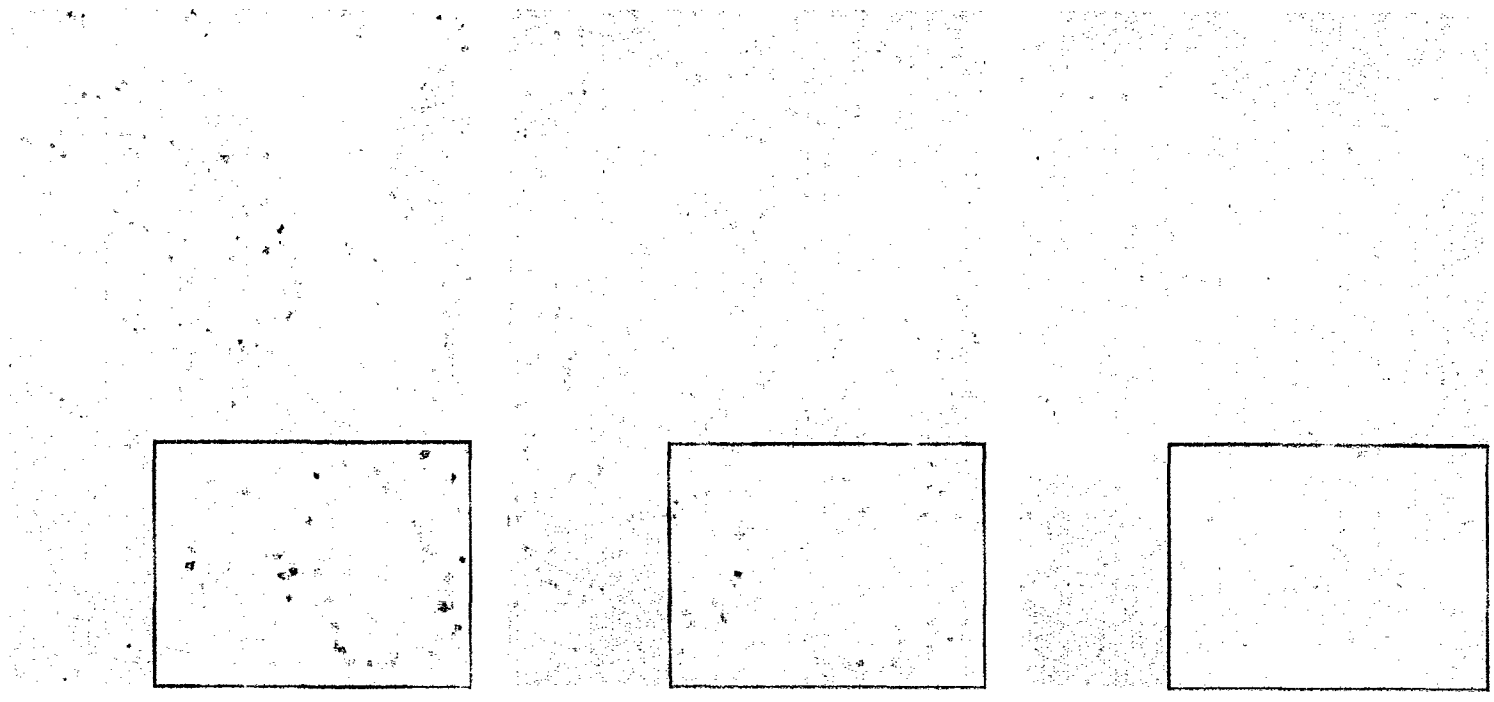
Figure 1. Percentage of proliferating cells in the virgin (50 days), pregnant (12.5 days), lactating (9 days) and involuting (2 days) mammary gland. Proliferation was determined by BrdU incorporation (n=6).

Figure 2. Mammary epithelial cells enter the cell cycle prior to the onset of apoptosis. A wave of cell proliferation, as determined by BrdU incorporation (n=3), occurred early in involution, and approximately 95% of these proliferating cells underwent apoptosis within 12-18 hours. All panels are at 200X magnification, and the insets are at 400X magnification.

Cell Proliferation in the Mammary Gland



Cell Proliferation During Mammary Gland Involution



Mammary Gland Development and Remodeling is Altered in the Caspase-$1^{-/-}$ Mouse.

Jane Wiesen and Zena Werb
Department of Anatomy
University of California, San Francisco, CA 94143-0452

Running Title: Development is Altered in the Caspase-$1^{-/-}$ Mammary Gland.

Keywords: caspase-1, apoptosis, cell proliferation, mammary gland, development

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Introduction

Apoptosis is an essential process whereby damaged or unwanted cells are removed via proteolytic digestion. Misregulation of the apoptotic pathway can lead to cancer, neurodegenerative diseases, and other pathological conditions (Thompson). A number of signals can induce cell death via the apoptotic pathway including hormone or growth factor withdrawal, signaling through Fas and tumor necrosis factor (TNF) receptors, ultraviolet (UV) and ionizing radiation, and loss of attachment to the extracellular matrix (ECM) (refs, Boudreau et al.). These different death inducing stimuli may use distinct signaling pathways. However, in each case, it is the caspase family of proteases which play a fundamental role in the downstream execution of the apoptotic pathway. The caspases compose the proteolytic cascade, which results in the cleavage of critical proteins involved in DNA repair (e.g. poly(ADP)-ribose polymerase, DNA-protein kinase) (Lazebnik et al., Song et al.), cell cycle control (e.g. Rb) (Janicke et al.), and maintenance of the cytoskeleton (e.g. fodrin, actin, lamins) (Martin et al., Rao et al., Mashima et al., Orth et al.).

Analysis of the phenotypes of knockout mice has provided vital information regarding the function of the caspases *in vivo*. Mice with a null mutation in the caspase-8 gene have abnormal heart development (Varfolomeev et al.), while caspase-$2^{-/-}$ mice have defective oocyte development (Bergeron et al.). Lack of caspase-3, caspase-9, or Apaf-1 gene expression results in abnormal development of the brain (Kuida et al., 1996, Woo et al., Kuida et al., 1998, Hakem et al., Yoshida et al., Cecconi et al.). The role of caspase-1 in the apoptotic pathway is unclear. Caspase-$1^{-/-}$ mice have impaired production of interleukin-1 alpha and beta (IL-1α and β), and caspase-$1^{-/-}$ thymocytes are resistant to Fas-

induced apoptosis, but otherwise, caspase-$1^{-/-}$ mice develop normally (Kuida et al., 1995, Li et al.). However, others have found that caspase-1 is involved in the neuronal cell death associated with ischemic brain injury, trophic factor withdrawal, toxicity, and neurodegenerative diseases such as ALS and Huntington's disease (Friedlander et al.[a,b], Klevenyi et al., Hara et al., Ona et al.) Moreover, inhibition of caspase-1 delays the onset and progression in a mouse model of Huntington's disease (Ona et al.). These knockout mouse models have demonstrated that despite the redundancy of function among the caspase family members, specific caspases are crucial for executing apoptosis in different tissues and organs.

To determine whether caspase-1 is involved in the induction of apoptosis *in vivo*, we examined mammary gland development, and remodeling in caspase-$1^{-/-}$ mice. The mammary gland undergoes extensive remodeling following the cessation of lactation and is an excellent example of a physiological process in which caspases mediate apoptosis. During this period of remodeling or involution, there is a dramatic increase in apoptosis in mammary epithelial cells (Strange et al., Lund et al.). We have previously shown that caspase-1, or interleukin-1β converting enzyme (ICE), is expressed in the involuting mammary gland (Lund et al.). Likewise, when apoptosis is induced by removal of the ECM in mouse mammary epithelial cells *in vitro*, caspase-1 is activated (Boudreau et al.). Additionally, we determined whether the apoptotic pathway was altered in the absence of caspase-1, by characterizing the percentage of cells undergoing apoptosis and proliferation during mammary development and remodeling in the caspase-$1^{-/-}$ mice. In this report, we show that the mammary glands of caspase-$1^{-/-}$ mice are

delayed in their remodeling, and that the rate of mammary development during pregnancy is modified as a result of the this alteration in the apoptotic pathway.

Materials and Methods

Animals

Caspase-$1^{-/-}$ mice were provided by John Mudgett at Merck Research Laboratories. Wild type mice of the same strain were used as controls and underwent identical treatments and analysis at each timepoint. After mating pairs were set up, vaginal plugs were checked daily to determine the day of pregnancy. To normalize the amount of suckling per lactating mouse, lactating mothers were left with 9 pups for a period of 9 days . To induce the onset of mammary gland involution, pups were removed after 9 days of lactation. Four to six mice were used at each timepoint to characterize morphology, gene expression, and rates of cell proliferation and apoptosis.

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10 different areas of sectioned mammary glands from at least 3 different mice at each timepoint.

To determine the relationship between proliferation and the onset of apoptosis in the mammary epithelial cells, pulse-chase experiments were performed in which BrdU was administered starting at 24 hours of involution, and re-administered every 6 hours for 24 hours to label the entire population of proliferating cells.

To analyze apoptosis, paraffin sections of the mammary glands were stained using the Apoptag Fluorescein kit (Oncor), and cells were counterstained with propidium iodide (Oncor). Cells undergoing apoptosis were visualized by fluorescence microscopy. The percentage of cells undergoing apoptosis was determined by counting at least 12 different areas of sectioned mammary glands from at least 3 different mice at each timepoint.

Results

The caspase-1 gene is expressed in the mouse mammary gland

To determine when the caspase-1 gene is expressed in the mouse mammary gland, we performed RT-PCR on RNA isolated from mammary glands collected during pregnancy, lactation, involution, and virgin development. We found that the caspase-1 gene was first expressed in mid-pregnancy (day 9) (Figure 1, lane 2). The caspase-1 gene was expressed throughout pregnancy (lanes 2-5), during lactation (lane 6), and during involution (lanes 7-11, 13). However, no caspase-1 gene expression was seen during virgin development (Figure 1, lanes 16-18).

Involution is altered in the caspase-1$^{-/-}$ mammary gland

Following the forced cessation of lactation, the mammary gland undergoes a rapid period of remodeling known as involution. After cessation of milk removal from the gland, milk continues to be secreted and accumulates in the alveolar lumina for 1-2 days. During this time, local factors induce the expression of cell cycle regulatory genes, ECM-degrading proteinases and their inhibitors in a coordinated and temporal pattern (ref). A subset of mammary epithelial cells are instructed to enter the cell cycle at day 2 of involution, which is followed by a large wave of apoptosis which peaks at day 4 of involution (ref, Figure 2). Furthermore, pulse-chase experiments revealed that of the luminal epithelial cells that were proliferating and therefore incorporating BrdU, approximately 95% of these cells underwent apoptosis within 12-18 hours (Figure 3). While many mammary epithelial cells are labeled at 47 hours of involution, 13 hours later (at 60 hours of involution) few labeled cells remain, and almost all labeled cells are lost 21 hours later (at 68 hours of involution) (Figure 3). This demonstrates that the mammary epithelial cells entered the cell cycle prior to the onset of apoptosis. Interestingly, parallel experiments which examined apoptosis during uterine regression revealed that in contrast to the mammary epithelial cells, uterine epithelial cells do not enter the cell cycle prior to the onset of apoptosis (Wiesen and Werb, unpublished). However, when we examined caspase-1$^{-/-}$ mammary glands, we found that this entry into the cell cycle, normally seen on day 2 of involution, was delayed (Figure 2).

As involution progresses, basement membranes are degraded by proteinases, the expression of milk protein genes fall, the alveolar structures collapse, the secretory luminal epithelial cells, the endothelial cells, and the myoepithelial cells are removed by apoptosis, and most

epithelial cells are replaced by adipose tissue (ref). We found that rate of involution was altered in caspase-$1^{-/-}$ mammary glands compared to the wild type glands. When we examined the morphology of the involuting mammary glands, we found that in the caspase-$1^{-/-}$ mammary glands the epithelial cells were replaced by adipocytes more rapidly than in the wild type glands (Figure 4).

Lactation is maintained in the caspase-$1^{-/-}$ mammary gland

To determine whether this alteration in the rate of involution seen in the caspase-$1^{-/-}$ mammary glands was influenced by any defect in lactation or lobulo-alveolar development, we examined the caspase-$1^{-/-}$ mammary glands during these developmental processes. We found that lactation was functionally normal in the caspase-$1^{-/-}$ mammary glands. The mammary epithelial cells underwent functional differentiation, as determined by the production of the milk protein beta-casein (data not shown). The lactating mothers were capable of supplying their pups with enough milk to support their growth and development, as the caspase-$1^{-/-}$ pups showed normal rates of weight gain and no increase in mortality (data not shown). These results demonstrate that lactation proceeds normally in the absence of caspase-1.

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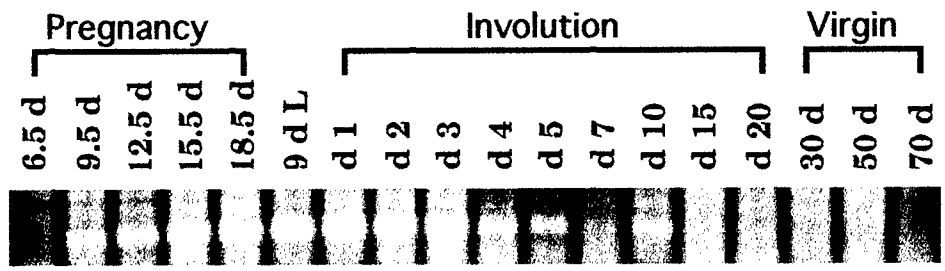
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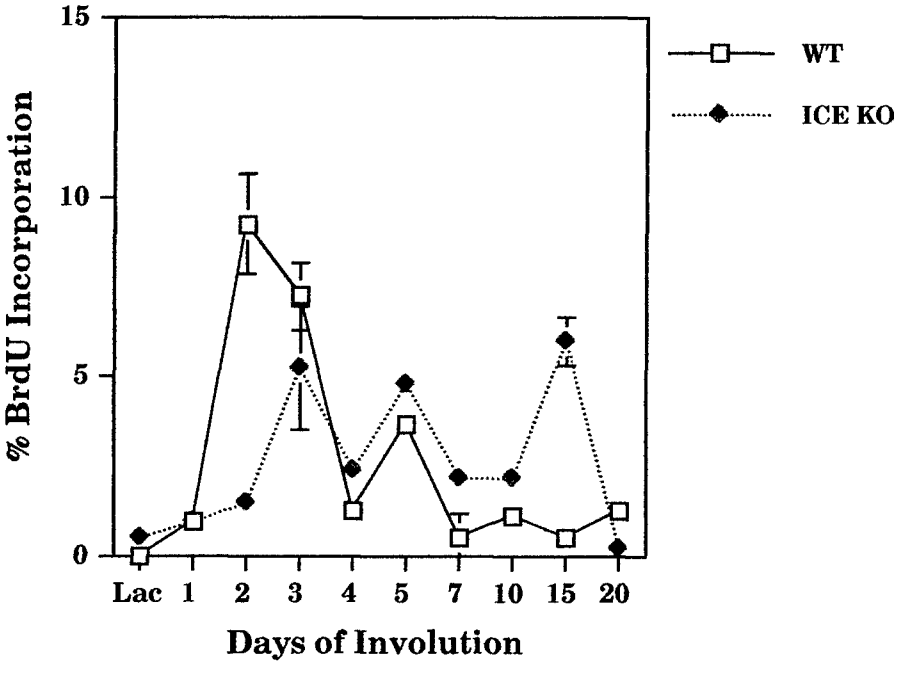
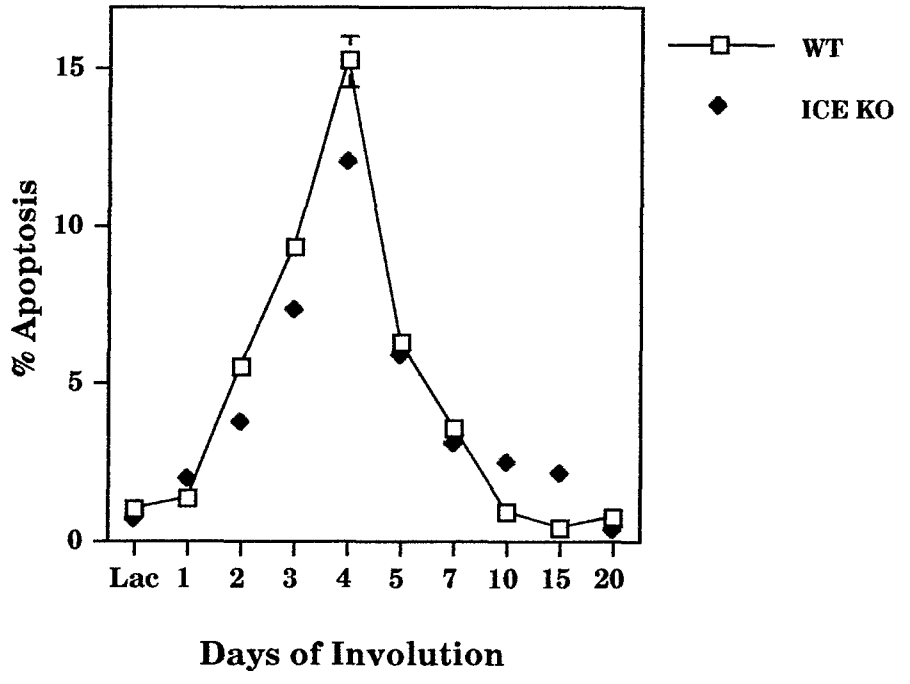
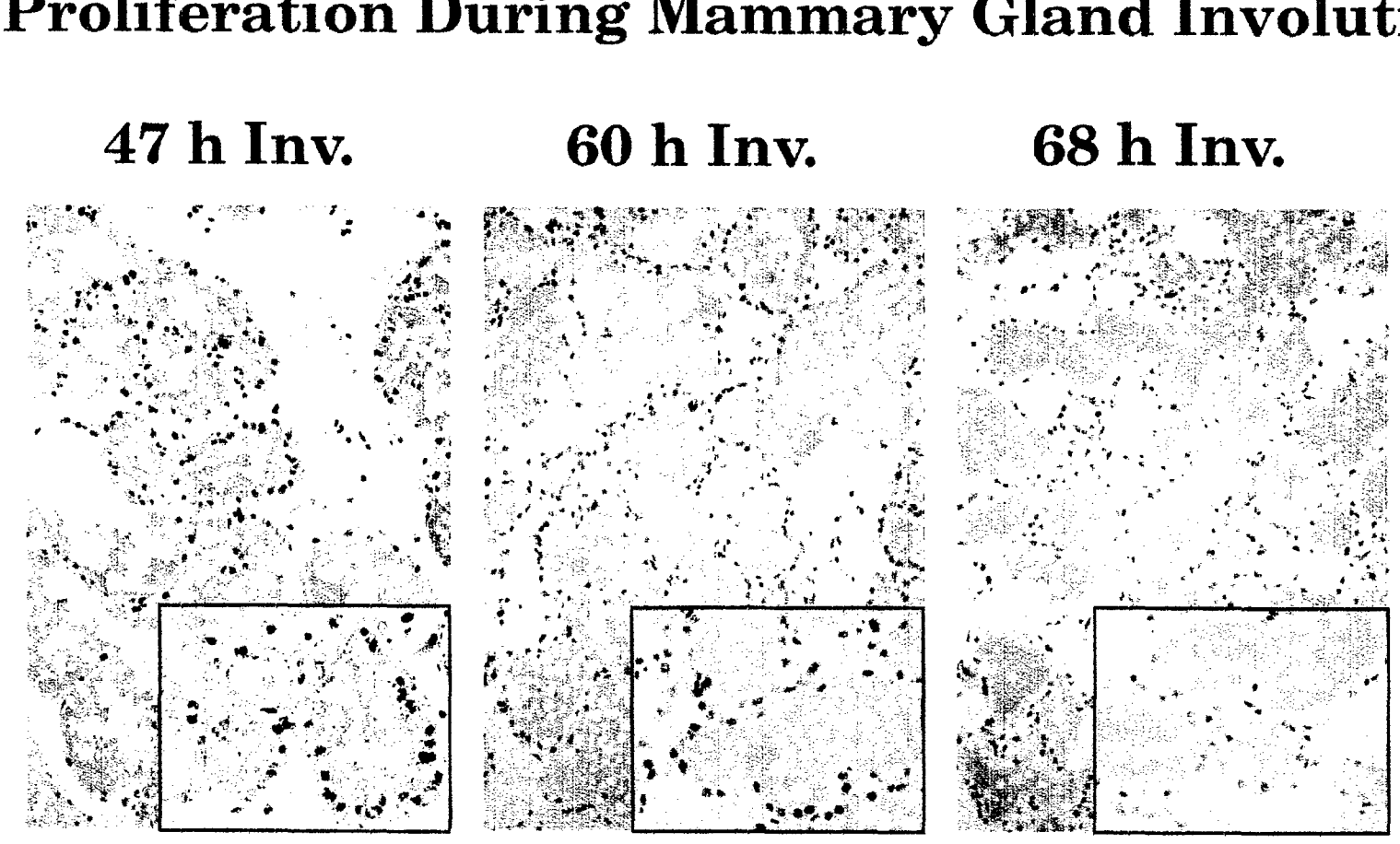
BrdU Incorporation during Involution

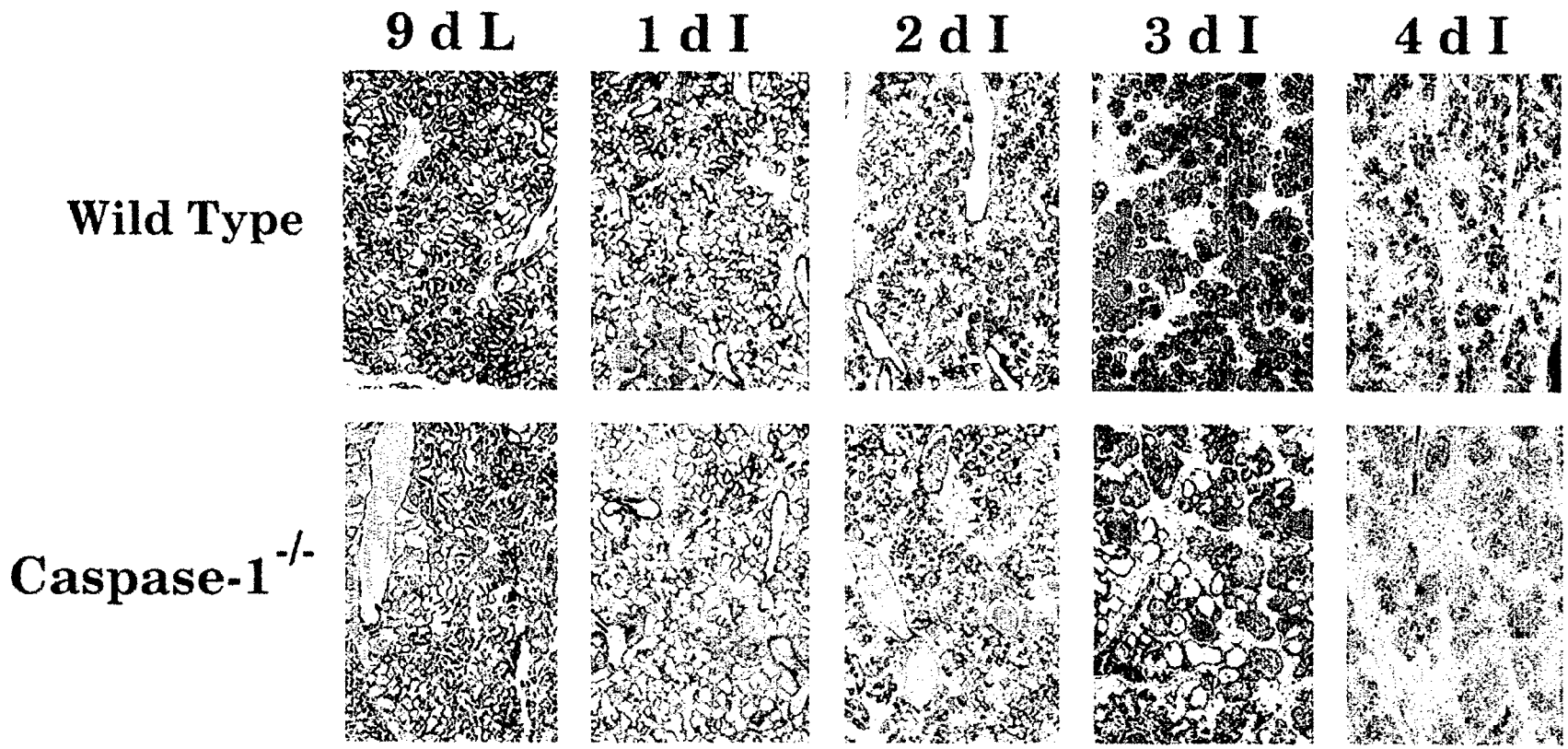


Apoptosis during Involution



Cell Proliferation During Mammary Gland Involution

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Wild Type
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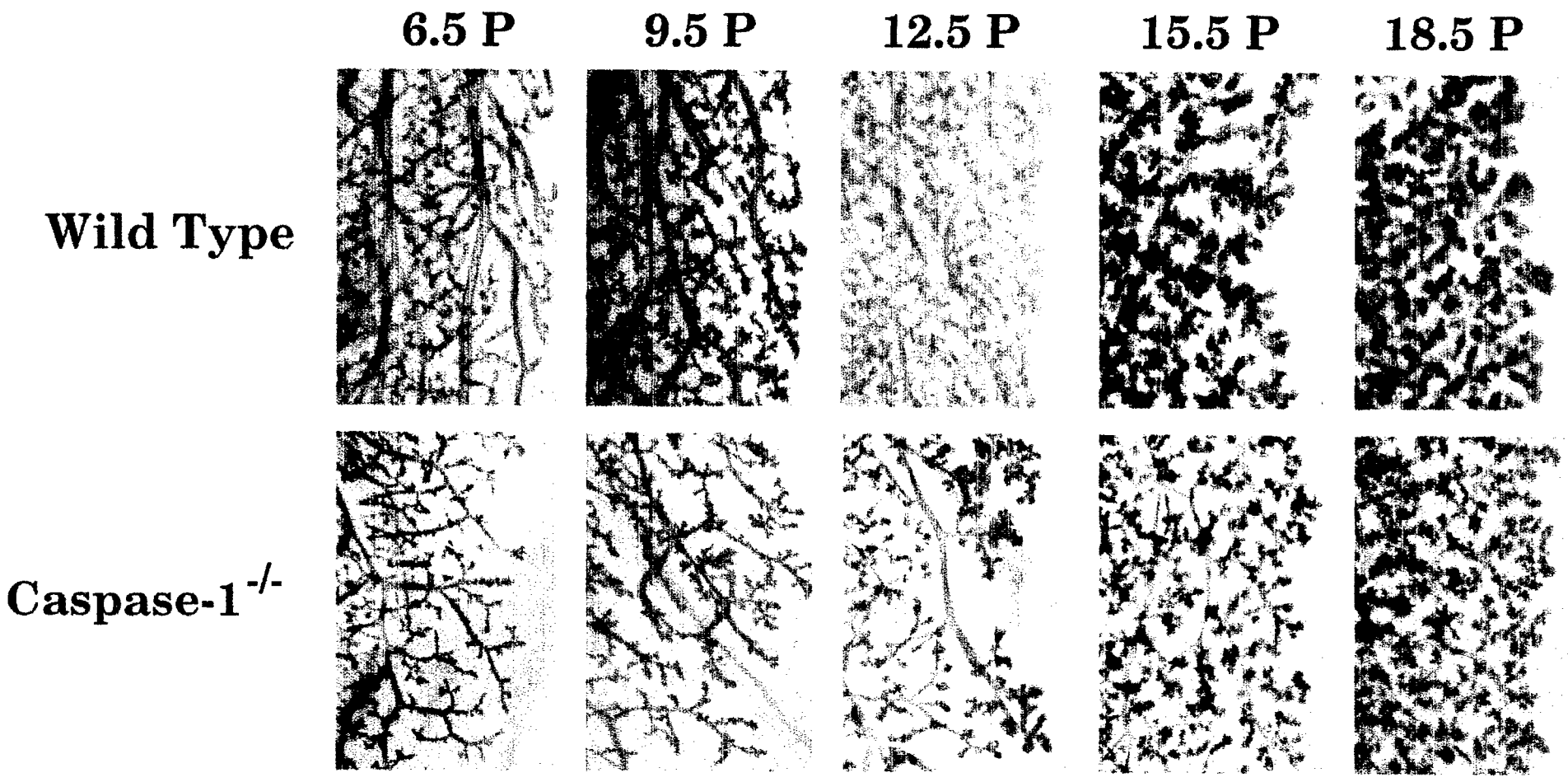
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Wild Type
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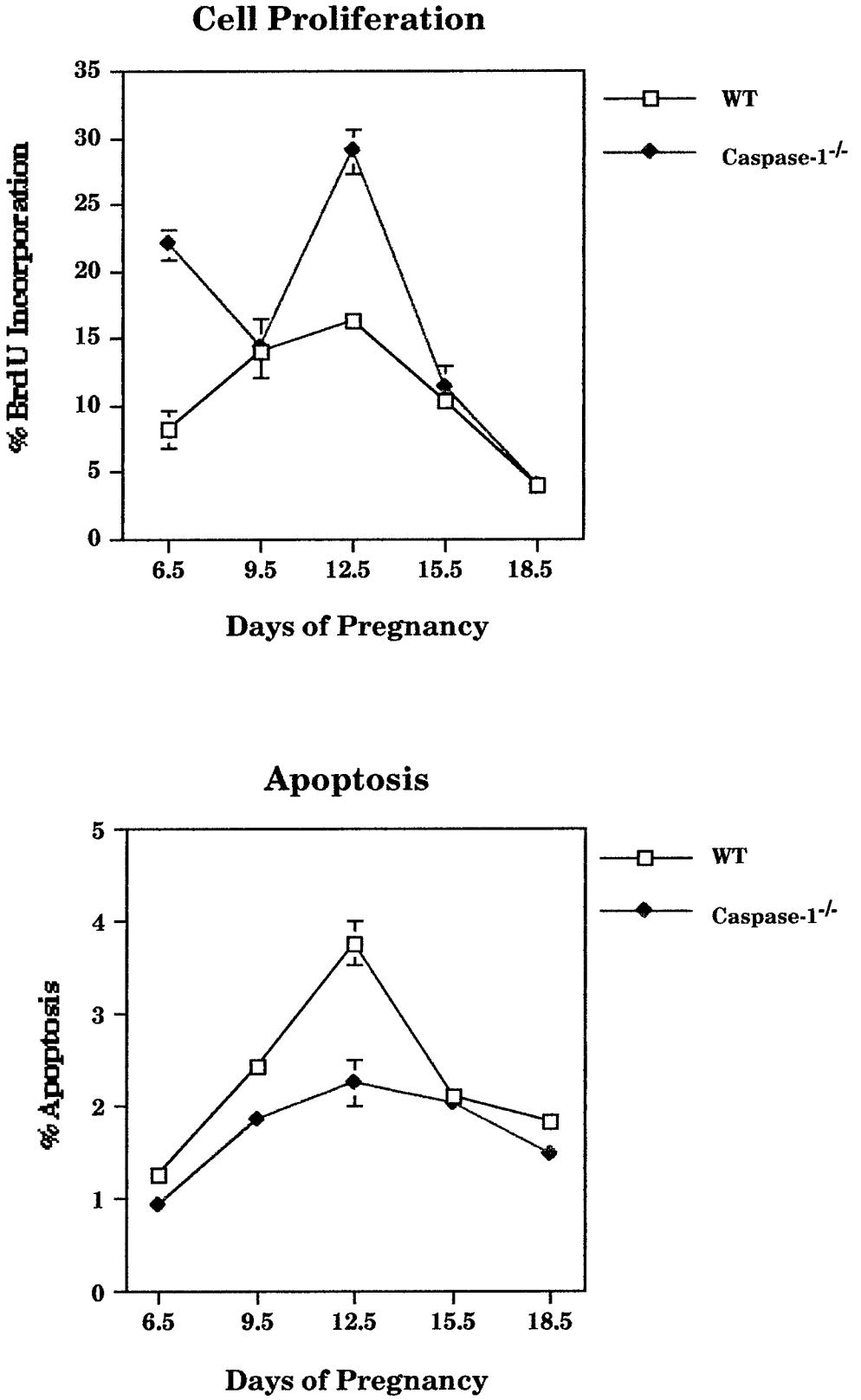
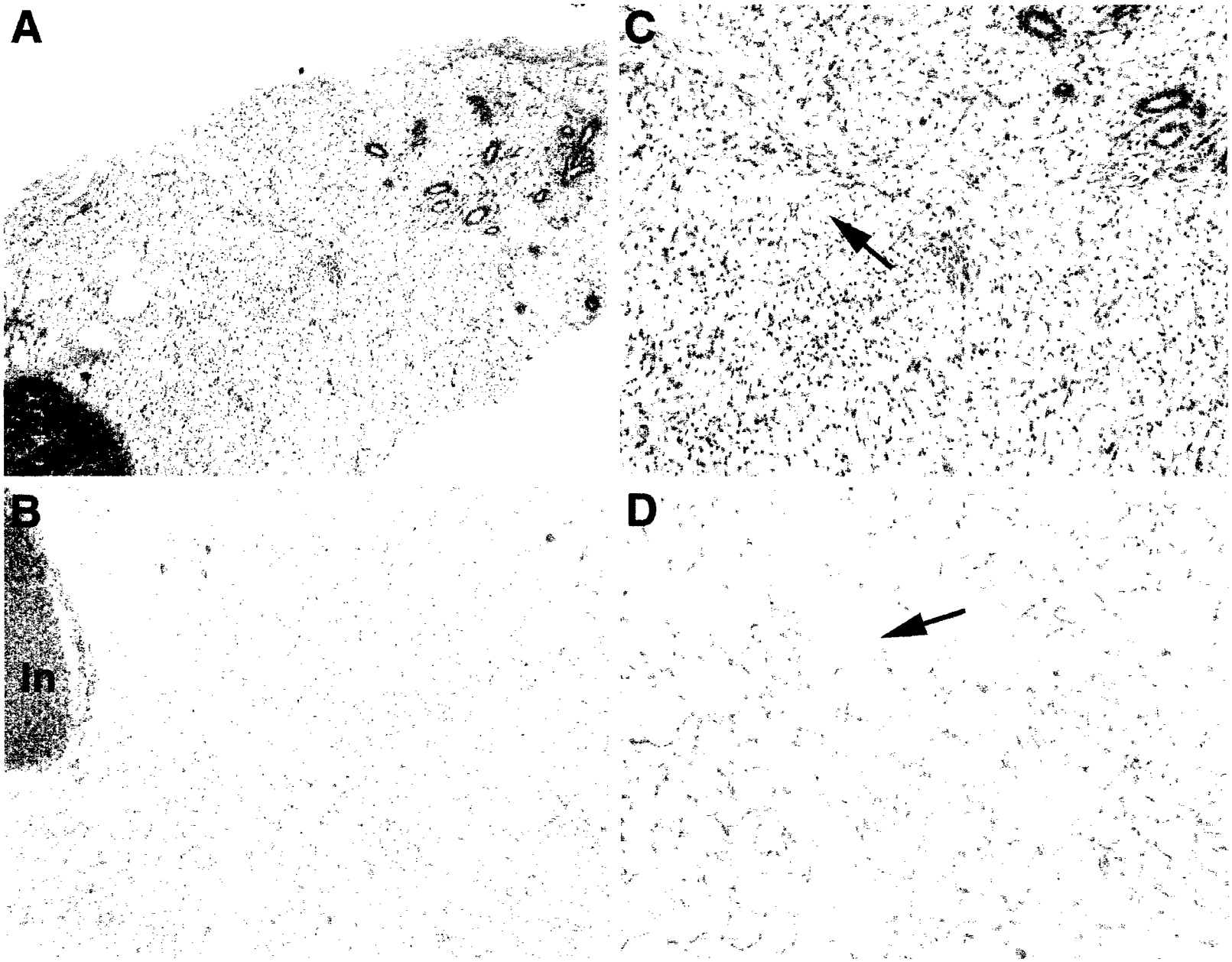


Figure 2. Percentage of Cells Undergoing Proliferation and Apoptosis in Wild Type or Caspase-1$^{-/-}$ Mouse Mammary Glands during Pregnancy. Data points represent the means ± SEM of labelled cells in 12 different fields from 4 different mice.

Figure 1: Hematoxylin and eosin stained sections of mammary gland tissue from MMP14 null (A and C) and wild type (B and D) pups at post-natal day 10. Arrows indicate adipose tissue, and ln indicates the lymph node. A and B are at 50X magnification. C and D are at 200X magnification.



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Experience

8/00-Present **Senior Scientist, Department of Pharmacology, Bayer Biotechnology, Berkeley, California.**

2/97-7/00 **Post-doctoral fellow-laboratory of Dr. Zena Werb, University of California, San Francisco, CA – Molecular Genetics**
Analyzing maternal angiogenesis and placenta development during pregnancy using the following knockout mouse models: gelatinase B, Timp-1, alpha V integrin, ets-2 and the EGF receptor. In addition to standard developmental biology techniques, I have utilized *in situ* hybridization, immunohistochemistry, matrix metalloproteinase and angiogenesis assays, tumor implantation into nude mice and other types of rodent surgery.

9/93-1/97 **Post-doctoral fellow-laboratory of Dr. Stanley J. Korsmeyer, Washington University School of Medicine, St. Louis, MO – Molecular Oncology**
Created a knockout mouse model to investigate the developmental role of the Bcl-2 family member Mcl-1. In addition to standard molecular biology techniques to clone and map the gene and create a targeting vector, I harvested mouse embryo fibroblasts, made embryonic stem cells, harvested, manipulated and cultured pre-implantation embryos, isolated and cultured inner cell masses, performed apoptosis assays, nested PCR, and histological analysis. Basic animal handling including breeding, injections, bleeding and perfusion were also performed.

4/88-5/93 **Graduate Student-laboratory of Dr. Marian E. Koshland (deceased), University of California, Berkeley, CA - Immunology**
Identified and characterized the function of a transcription element located within the J chain promoter and a transcription factor that bound to it using the following techniques: methylation protection footprinting, copper phenanthroline footprinting, nuclear extract preparation, plasmid construction, sequencing, site directed mutagenesis, transient transfection of mammalian cells, *in vitro* transcription and translation, and PCR amplification.

Education

Post-doctoral fellow, Department of Anatomy, University of California-San Francisco, February 1997 to present.
Post-doctoral fellow, Division of Molecular Oncology, Howard Hughes Medical Institute, Washington University in St. Louis, September 1993 to January 1997.
Ph. D. in Immunology, University of California-Berkeley, May 1993.
B. S. in Honors Biology, University of Illinois-Champaign-Urbana, May 1987.

Julie L. Rinkenberger

Awards

American Cancer Society Post-doctoral fellowship June 1995-May 1998.
Outstanding Graduate Student Instructor, University of California-Berkeley, 1991.
Anderson-Kelso Award for Departmental Highest Distinction, School of Life Sciences, University of Illinois, 1987.
School of Life Sciences Distinction Award, Second Place, University of Illinois, 1987.

Memberships in Professional Organizations

Associate Member of the American Association for Cancer Research.

Publications

Julie Rinkenberger and Zena Werb. (2000) The labyrinthine placenta. Nature Genetics, 25, 248-250.

Julie L. Rinkenberger, Susan Horning, Barbara Klocke, Kevin Roth and Stanley J. Korsmeyer. (2000) Mcl-1 deficiency results in peri-implantation embryonic death. Genes & Development, 14, 23-27.

Jeffrey J. Wallin, **Julie L. Rinkenberger**, Sulekha Rao, Edwin R. Gackstetter, Marian E. Koshland, and Patty Zwollo. (1999) B cell-specific activator protein prevents two activator factors from binding to the immunoglobulin *J Chain* promoter until the antigen-driven stages of B cell development. Journal of Biological Chemistry, 274, 15959-15965.

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R. E. Lloyd, **J. L. Rinkenberger**, B. A. Hug, and R. W. Tuveson. (1990) Growing *Escherichia coli* mutants deficient in riboflavin biosynthesis with non-limiting riboflavin results in sensitization to inactivation by broad-spectrum near-ultraviolet light (320-400nm). Photochemistry and Photobiology, 52, 897-901.

Julie L. Rinkenberger

Invited Lectures

"Making Connections for Survival During Murine Implantation" for the Reproductive Endocrinology Center Seminar Series, University of California, San Francisco, March 6, 1998.

"Extracellular Matrix Remodeling, Invasion, and Apoptosis During Murine Implantation" for the Society for the Study of Reproduction, Minisymposium XII on Extracellular Matrix and Cell Adhesion Molecules, Thirtieth Annual Meeting, August 2-5, 1997.

Meeting Talks

"Deletion of the Bcl-2 Family Member, Mcl-1, by Gene Targeting Results in Peri-implantation Embryonic Lethality" for the Programmed Cell Death meeting at Cold Spring Harbor Laboratories, September 17-21, 1997.

Posters

"Deletion of Gelatinase B Results in Compromised Embryo Implantation", presented at the 57th Society for Developmental Biology Annual Meeting, Stanford University, June 20-25, 1998.

"Deletion of Gelatinase B Inhibits Maternal Angiogenesis in Early Pregnancy", presented at the Keystone Symposia on Angiogenesis and Vascular Remodeling, March 28-April 2, 1998.

"Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases in the Stroma Regulate Mammary Ductal Morphogenesis", presented at the DoD Breast Cancer Research Program Era of Hope Meeting, June 8-12, 2000.

References

Available upon request.